CHEMICAL CONSTITUENTS OF *PHOEBE GRANDIS* (NEES) MERR, *PHOEBE TAVOYANA* (MEISSN.) HK.F., AND *ACTINODAPHNE SESQUIPEDALIS* HOOK. F. VAR. GLABRA AND THEIR BIOLOGICAL ACTIVITIES

HANITA BINTI OMAR

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HANITA BINTI OMAR

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ABSTRACT

Chemical Constituents of *Phoebe grandis* (Nees) Merr, *Phoebe tavoyana* (Meissn.) Hk.F., and *Actinodaphne sesquipedalis* Hook. F. var. glabra And Their Biological Activities

Twenty extracts of *Phoebe grandis* and *Phoebe tavoyana*; and *Actinodaphne sesquipedalis* were underwent preliminary screening for their cytotoxicity, antioxidant, antibacterial and antiplasmodial activities. The potential extracts were chosen for detailed phytochemical investigation involving isolation of compounds by chromatographic methods, structural elucidation by means of spectroscopic methods such as UV, IR, MS, 1D and 2D NMR (COSY, HMQC, HMBC, NOESY) and also by comparison with the literature. Further bioassay screenings for some of the identified compounds were also carried out. These techniques have led to the isolation and identification of several isoquinoline type such as aporphine, proaporphine, oxoaporphine, benzylisoquinolines; morphinandienone and sterols.

The crude extracts and some of the isolated compounds were screened for cytotoxic, antioxidant and antibacterial activity, using MTT (Microculture Tetrazolium salt), DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (ferric reducing ability of plasma) and disc diffusion methods, respectively. The cell lines used in the cytotoxic assay were MCF-7 (human estrogen receptor (ER+) positive breast cancer), Caov-3 (Human Ovarian cancer cell line) and HepG2 (Human Liver cancer). The antibacterial activity was tested against selected pathogenic bacteria *Bacillus subtilis* (gram-positive), *Staphylococcus aureus* S1434 (gram-positive), *Staphylococcus epidermidis* (gram-positive), *Escherichia coli* (gram-negative), *Salmonella typhi* (gram-negative), *Pasteurella multocida* (gram-negative), *Enterobacter cloacae* (gram-negative) and Methicillin resistant *Staphylococcus aureus* (MRSA) (gram-positive).

Phytochemical study on *P. grandis* leaves has led to the isolation of two new proaporphine alkaloids litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**) along with two known oxoaporphine lysicamine (**54**) and dicentrinone (**58**); one known proaporphine hexahydromecambrine A (**57**). Interestingly, all of compounds were reported for the first time present in the leaves of *P. grandis*. Phytochemical work of *P. grandis* bark yielded β-sitosterol (**59**), stigmasterol (**60**), boldine (**5**), N-methyllaurotetanine (**51**), reticuline (**61**) and laurolitsine (**6**). However, investigation of the leaves of *P. tavoyana* afforded seven alkaloids of which two were new compounds; tavoyanine A (**63**) and tavoyanine B (**64**) along with four known aporphines; laetanine (**62**), roemerine (**20**), laurolitsine (**6**) and boldine (**5**); and one morphinandienone, sebiferine (**22**). Phytochemical study has also been performed on *A. sesquipedalis* leaves and has yielded eight compounds, β-sitosterol (**59**), dicentrine (**52**), N-methyllaurotetanine (**51**), stigmasterol (**60**), dicentrinone (**58**), boldine (**5**), norisocorydine (**65**) and laurolitsine (**6**); and another five compounds have been isolated from the fruits which consists of three alkaloids were dicentrine (**52**), liriodenine (**19**) and dicentrinone (**58**) and two sterols were β-sitosterol (**59**) and stigmasterol (**60**). All compounds, except dicentrine (**52**) have been isolated for the first time from *A. sesquipedalis*.

Lysicamine (**54**) and litsericinone (**55**), exhibited cytotoxic activity against MCF7 and HepG2 cell lines. While, 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) exhibited cytotoxic activity against the HepG2 cell line. 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) were not toxic towards the MCF7 cell line. Lysicamine (**54**) also displayed a strong antibacterial activity against *Staphylococcus aureus* with inhibition zones of 13.33 ± 0.57 mm. While, roemerine (**20**), laurolitsine (**6**), boldine (**5**) and sebiferine (**22**) displayed significant inhibition activity against *P. falciparum* (3D7).

ABSTRAK

Kandungan Kimia *Phoebe grandis* (Nees) Merr, *Phoebe tavoyana* (Meissn.) Hk.F., dan *Actinodaphne sesquipedalis* Hook. F. var. glabra Dan Aktiviti Biologinya.

Dua puluh ekstrak dari *Phoebe grandis* dan *Phoebe tavoyana*; dan *Actinodaphne sesquipedalis* telah menjalani penyaringan awal untuk sitotoksik, antioxidan, antibakteria dan antiplasmodial aktiviti. Ekstrak berpotensi dipilih untuk siasatan fitokimia terperinci melibatkan pengasingan sebatian dengan kaedah kromatografi, penentuan struktur organik melalui kaedah spektroskopi seperti UV, IR, MS, 1D dan 2D NMR (COSY, HMQC, HMBC, NOESY) dan juga oleh perbandingan dengan kesusasteraan. Kajian bioassai lanjut untuk beberapa sebatian yang dikenalpasti juga dijalankan. Teknik-teknik ini telah membawa kepada pemencilan dan pengenalpastian beberapa sebatian jenis isokuinolina seperti aporfina, proaporfina, oxoaporfina, benzilisokuinolina; morfina dan sterol.

Ekstrak-ekstrak mentah dan sebatian-sebatian yang dipencilkan telah diuji aktiviti sitotoksik, antioxidant dan antibacterial aktiviti dengan menggunakan kaedah MTT (garam Mikrokultur Tetrazolium), DPPH (1,1-difenil-2-pikrilhidrazil), FRAP (ferric mengurangkan keupayaan plasma) dan peresapan cakera. Sel-sel yang digunakan untuk ujikaji sitotoksik adalah sel kanser payudara reseptor positif estrogen (ER+) manusia (MCF7), sel kanser ovari manusia (Caov-3) dan hepatokarsinoma manusia (HepG2). Aktiviti antibakteria telah diuji ke atas bakteria patogenik yang telah dipilih seperti *Bacillus subtilis* (gram-positive), *Staphylococcus aureus* S1434 (gram-positive), *Staphylococcus epidermidis* (gram-positive), *Escherichia coli* (gram-negative), *Salmonella typhi* (gram-negative), *Pasteurella multocida* (gram-negative), *Enterobacter cloacae* (gram-negative) and Methicillin resistant *Staphylococcus aureus* (MRSA) (gram-positive).

Kajian fitokimia ke atas daun *P. grandis* telah menghasilkan pemencilan dua terbitan baru alkaloid proaporfina litsericinon (**55**) and 8,9,11,12-tetrahydromecambrina (**56**) di samping dua oxoaporfina yang dikenalpasti dan sering ditemui lysicamina (**54**) dan dicentrinona (**58**); dan satu proaporfina hexahydromecambrine A (**57**) yang sudah dikenalpasti. Menariknya, sebatian-sebatian ini dilaporkan pertama kali wujud dalam daun *P. grandis*. Kajian fitokimia ke atas kulit pokok *P. grandis* menghasilkan β-sitosterol (**59**), stigmasterol (**60**), boldina (**5**), N-methyllaurotetanina (**51**), retikulina (**61**) dan laurolitsina (**6**). Namun begitu, kajian terhadap daun *P. tavoyana* memberikan tujuh alkaloid yang mana dua adalah sebatian baru tavoyanina A (**63)** dan tavoyanina B (**64)** di samping empat aporfina yang dikenalpasti; laetanina (**62**), roemerina (**20**), laurolitsina (**6**) dan boldina (**5**); dan satu morphinandienon, sebiferina (**22**). Kajian fitokimia juga dijalankan ke atas daun *A. sesquipedalis* dan telah menemui lapan sebatian menarik β-sitosterol (**59**), dicentrina (**52**), N-methyllaurotetanina (**51**), stigmasterol (**60**), dicentrinona (**58**), boldina (**5**), norisocoridina (**65**) dan laurolitsina (**6**); dan lima sebatian telah dipencilkan daripada buah yang mengandungi tiga alkaloid iaitu dicentrina (**52**), liriodenina (**19**) dan dicentrinona (**58**) dan dua sterol iaitu β-sitosterol (**59**) dan stigmasterol (**60**). Semua sebatian, kecuali dicentrina (**52**) adalah pemencilan buat pertama kali dari *A. sesquipedalis*.

Lysicamina (**54**) and litsericinon (**55**), menunjukkan aktiviti sitotoksik terhadap sel kanser MCF7 dan HepG2. Manakala, 8,9,11,12-tetrahydromecambrina (**56**) and hexahydromecambrina A (**57**) menunjukkan aktiviti sitotoksik terhadap sel kanser HepG2. 8,9,11,12-tetrahydromecambrina (**56**) dan hexahydromecambrina A (**57**), tidak toksik terhadap sel kanser MCF7. Lysicamina (**54**) juga mempamerkan aktiviti antibakteria kuat terhadap *Staphylococcus aureus* dengan zon perencatan 13.33 ± 0.57 mm. Manakala, roemerina (**20**), laurolitsina (**6**), boldina (**5**) and sebiferina (**22**) telah menunjukkan aktiviti perencatan yang signifikan terhadap *P. falciparum* (3D7).

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CHAPTER 3: METHODOLOGY

LIST OF SCHEMES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

α	Alfa
β	Beta
δ	Delta value (chemical shift)
λ_{max}	Maximum wavelength
Å	Angstrom
ATCC	American Type Culture Collection
^{13}C	13-Carbon
CAOV-3	Human Ovarian cancer cell line
C_6H_{12}	Hexane
$CHCl_3$	Chloroform
CC	Column Chromatography
CGM	Complete Growth Media
CH_3	Methyl group
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CH_2Cl_2	Dichloromethane
CH_3COCH_3	Acetone
COSY	H-H Correlation Spectroscopy
d	Doublet
dd	Doublet of doublet
ddd	Doublet of doublet of doublet
dt	Doublet of triplet
DEPT	Distortioness Enhancement by Polarisation Transfer
DMSO	Dimethyl sulphoxide

DOX	Doxorubicin
DPPH	1,1’-diphenyl -2-picrylhydrazyl
EtOAC	Ethyl acetate
ESI	Electrospray Ionization
EIMS	Electron Impact Mass spectroscopy
FCS	fetal calf serum
FT-NMR	Fourier Transform NMR
g	Gram
GCMS	Gas Chromatogram-Mass Spectrometry
^{1}H	Proton
HCl	Hydrochloric acid
HepG2	Human Liver cancer
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HREIMS	High Resolution Electron Ionization Mass Spectral
Hz	Hertz
IC_{50}	Inhibition Concentration at 50 percent
Id	Inhibition diameter
IR	Infrared
J	Coupling constant (Hz)
m	multiplet
m	Metre
m/z	Mass/charge ratio
MHz	Mega Hertz
ml	Mililitre
MS	Mass Spectrum

Mg/ml^{-1}	Microgram per mililitre
MeOH/ CH_3OH	Methanol
m.p	Melting point
MCF-7	Human Breast cancer cells
MS	Mass Spectrum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
m/z	Mass per charge
nm	Nanometre
NH_3	Ammonia
NMR	Nuclear Magnetic Resonance
1D NMR	One dimensional NMR
2D NMR	Two dimensional NMR
NOE	Nuclear Overhauser Effect
OCH_3	Methoxyl group
OCH_2O	Methylenedioxy group
OH	Hydroxyl group
OD	Optical density
PBS	Phosphate buffered saline
ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography
ROS	Reaction oxygen species
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
TLC	Thin Layer Chromatography
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 General Historical Aspects

For more than a thousands years, natural products have been recognised as playing a very important role throughout the world in treating and inhibiting human diseases. Natural product medicines have been derived from various sources including marine organism, terrestrial microorganisms, terrestrial plants, terrestrial vertebrates and invertebrates (Newman *et al.*, 2000). Usually, the natural product has some biological activities for the use in pharmaceutical drug discovery and drug design. These bioactive compounds are known as the active principles and can act as a lead compounds.

Since time immemorial, historians from all over the world have known that apparently primitive peoples used herbs in often sophisticated ways. The ancient uses of plant materials as medicines can be found in archeological finds, history books and old literature. In fact, there are 20 plants are mentioned as medicinal agents to treat various ailment in the Holy Quran and 125 plants are in the Holy Bible (Musselman). Both the Quran (Dawood) and the Bible (Zumla & Lulat, 1989) include plants that have long been used for medicine. Nowdays, the efficacy of these plants has been documented with modern science.

More than 35,000 plant species have been reported to have been exploited various human cultures around the world for medical purposes (Lewington, 1993). However,

the number could be much higher as knowledge on the indigenous uses of plants was mostly passed on verbally from one generation to another and has largely remained undocumented. It is challenging to obtain information from practitioners of traditional medicine unless a genuine long term relationship has been made. Burkill (1966), in his extensive compilation of the economic products of the Malay Peninsular, recorded that not less than 1,300 plants have been used in traditional medicine.

1.2 Natural Products Research in Malaysia

Malaysia widely-recognised as one of the centres of tropical forest biological diversity, is richly endowed with flora and fauna, and microbial genetic resources. If exploited and managed wisely, these genetic resources could provide useful renewable products for not only the present but also the future generations (Soepadmo, 1999).

Malaysia endowed with thick tropical rain forests contains a large number of interesting pharmacologically active constituents and many more still waiting to be discovered. A tremendous amount of work needs to be done with our own forest in seeking and identifying potential plants for numerous medicinal uses for the benefit of humankind. Hence, there is a need for documentation of the plants from a taxonomic and ethnobotanical point of view. Besides, more appropriate, low cost and effective assays are required for plant screening phytochemical laboratories and certainly many interesting activities yet to be evaluated and discovered from our own heritage.

However, many obstacles in the course of the development of a compound as a drug because it is financially risky and time consuming as the drug would have to undergo a series of clinical trials to provide knowledge about its toxicity as well as its safety with

less side effects, stability, absorption, distribution and elimination from human body. Although many challenges will be faced, but with support from government especially in a developing country and also UNESCO in the early 70s, funds were made available for training of young scientists and regional cooperation became more coordinated where networking was encouraged. Malaysia was actively involved in the Regional Network for the Chemistry of Natural Products in Southeast Asia, and through this network, our scientists were exposed to current trends in the natural products area by interacting with other networks, notably from Australia (NCBINP), Sweden (IFS), and Pakistan (ISESCO). Therefore, intensive and extensive research works could be performed, especially in the search for more bioactive compounds and many promising natural wonder drugs can also be developed.

The earlier publications concentrated more on phytochemical work especially on alkaloids, (Kiang *et al.*, 1961) terpenes, phenils, alcohols, tannins, saponins, acids and the trend has continued over the years until the present time (Chan *et al*., 1969; Goh *et al.*, 1985; Kam *et al.*, 1993; Kam *et al.*, 1996; Rahmani *et al.*, 1985; Rahmani *et al.*, 2004; Mukhtar *et al.*, 2009; Ravikumar *et al.*, 2011; Omar *et al*., 2013; and Elya *et al.*, 2014). In a way, University of Malaya has gained a lot from their successful collaboration with the Institut de Chimie des Substances Naturelles (CNRS-France) which started in 1982 and since renewed in 1993. Research has been supported by the government with the grant IRPA (Intensification of Research in Priority Areas) programme in 1985. IRPA is its third term (1996 - 2000) and one of the areas that have been identified as a priority is the *commercialisation of biotechnology,* which also takes into account the development and production of biopharmaceuticals from plant genetic resources. Therefore, after the IRPA, the government still do support the research

through the funding given to the universities such as HIR- High Impact Research Grant (2009 - 2016).

Many in the University of Malaya are working diligently in the investigation of many interesting plant species such as those from Annonaceae, Apocynaceae, Lauraceae, Menispermaceae, and Rubiaceae family. They are searching not only for chemical varieties but also potential bioactive compounds. In continuation of the investigation on Malaysian local plants, I would like to report phytochemical work and biological activities of *Phoebe tavoyana*, *Phoebe grandis* and *Actinodaphne sesquipedalis*. The extracts of the plants were subjected to several series of chromatographic techniques to obtain the chemical constituents, as well as potential bioactive compounds.

1.3 Research Objectives

Research conducted for this study was based on the following objectives:

(a) To screen, evaluate and investigate the extracts of stem bark and leaves of *Phoebe grandis and Phoebe tavoyana;* and leaves, bark and fruits of *Actinodaphne sesquipedalis* for antibacterial, cytotoxic (MTT) and free scavenging activities using DDPH and Ferric reducing antioxidant potential (FRAP) assays.

(b) To isolate chemical constituents from the stem bark and leaves of *Phoebe grandis and Phoebe tavoyana*; from the leaves and fruits of *Actinodaphne sesquipedalis* using various chromatographic techniques.

(c) To characterise and elucidate the structure of the isolated compounds using spectroscopic methods -1D NMR such as ^{1}H NMR, ^{13}C NMR, DEPT and NOE DIFF; 2D NMR - COSY, HSQC, HMBC and NOESY; UV, IR and MS.

(d) To evaluate the biological activities of the isolated compounds for cytotoxic (MTT), antibacterial and antiplasmodial activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Traditional Medicinal Plants and the Development of Modern Medicine.

Plants, especially those with ethnopharmacological uses, have been the primary sources of medicines for early drug discovery. The extensive records from Traditional Chinese and Ayurveda were as sophisticated as western medicinal records, although they might use different methods or paradigms. For example as the records indicate the response to Artemisia preparations for malaria also provided the clue to the novel antimalarial drug artemisinin (also known as Qinghaosu) from *Artemisia annua*. Artemisinin has been in the frontline treatment for malaria since the late 1990s and has saved countless lives, especially among the world's poorest children (Miller & Su, 2011).

Many plants have been employed in folk medicine for their interesting bioactivities. For example, the bark of *Cinnamomum cassia* Blume is a very famous traditional medicine that has been widely used in Asian countries. The extracts from *C. cassia* have been claimed to reduce inflammation (Lee & Shibamoto, 2002), and to decrease serum glucose, platelet counts and total cholesterol (Khan *et al.*, 2003). Dhuley (1999) showed that cinnamon displays antioxidant activity in rats fed a high-fat diet. *Cinnamomum camphora* (L.) Presl is a major source of camphor which is taken orally to calm hysteria, nervousness, neuralgia and to treat serious diarrhea. Camphor is also known to be effective in treating colds and chills (Lee *et al*., 2006).

The opium poppy (an active principle is morphine) known in Ancient Egypt and the *Solanaceae* plants (active principles atropine) in ancient Greece have the therapeutic properties. The snakeroot plant was well regarded in India (active principle reserpine). Reserpine was the first compound shown to be an effective antidepressant in a randomised placebo-controlled trial (Davies & Shepherd, 1955). The herbalist in medieval England used extracts from the willow tree (salicin) as an anti-inflammatory agent (Bensky *et al.*, 2004). The Aztec and Mayan cultures of Mesoamerica used extracts from a variety of bushes and trees including the *ipecacuanha* root (active principle emetine), coca bush (active principle cocaine) and *Cinchona* bark (active principle quinine) (García *et al.*, 1999) were used to treat the symptoms of malaria long before the disease was identified. Pepper was used in Europe as a spice as early as the eleventh century. It was once employed in the treatment of gonorrhoea and chronic bronchitis but in large quantities was used as a condiment. Various species of *Ephedra* (*Ma-huang*) was known to the Chinese over 5000 years ago and ephedrine was isolated in 1887, it only came into extensive use during the last century.

In the early 1900s, approximately about 80% of all medicines were obtained from roots, barks and leaves. Fluid extracts were used frequently as medicine and people belief that for every ill there exist a cure in the plants of field and forest (Newman *et al.*, 2003). At recent times, natural products have continued to be significant sources of drugs and leads. Their dominant role is indicated by the fact that approximately 60% of anticancer compounds and 75% of drugs for infectious diseases are either natural products or natural product derivatives (Newman *et al.*, 2003; Cragg *et al.*, 2011). Despite of this success, research into natural products has experienced a steady global decline. Through synthesis and combinatorial chemistry there was unlimited supply of compound

libraries has greatly contributed to this declining interest in the screening of natural products by the pharmaceutical industry.

Natural products discovery and development have several opportunities in the field of pharmaceuticals, nutraceuticals, cosmetics, agrochemicals and fine chemicals. The requirements for the discovery, development and commercialisation of pharmaceuticals are generally well known. The required time for development of the pharmaceutical drugs can ranges from a few years to 20 years (McChesney *et al.*, 2007). For instance, the chemical structure of paclitaxel (Taxol®) was reported and identified as the cytotoxic active constituent of extracts of the bark of the Pacific yew tree *Taxus brevifolia* (Wani *et al.*, 1971). Taxol® was approved for marketing as a cancer chemotherapeutic agent at the end of 1992 which is 20 years later after its discovery. It is a potent anticancer drug with proven activity against a number of human solid tumours and has become standard treatment as the single agent or in combination chemotherapy for the management of advanced breast, ovarian and non-small-cell lung cancer (Wood *et al.*, 1995). On average, at least one decade is needed for the development and commercialisation of a new pharmaceutical discovery.

2.2 Natural Compounds for Cancer Treatment and Prevention

Natural products play a relevant role in cancer therapy today with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products from various sources such as plants, animals and microorganisms (also of marine origin). Large-scale anticancer drug discovery and screening programs such as those promoted by the National Cancer Institute (NCI) have played an important role in the development of anticancer natural compounds.

On recent times, more interest has been paid to protecting foods and human beings against oxidative damage caused by free radicals like hydroxyl, peroxyl, and superoxide radicals. One possible solution is to explore the potential antioxidant and anticancer properties of plant extracts or isolated products of plant origin (Namiki, 1990). There is an abundance of evidence that regular consumption of fruits and vegetables is associated with reduced risks of chronics diseases, such as cancers and cardiovascular disease (Doll, 1990); Dragsted *et al.*, 1993) which have been demonstrated to have positive effects in cancer therapy. For example, it was recently demonstrated that the green tea antioxidant EGCG (epigallocatechin-3-gallate) significantly slowed breast cancer growth in female mice (Nobili *et al.*, 2009). Fruits and vegetables (plant materials) are a primary food source providing essential nutrients for sustaining life; they also contain a variety of phytochemicals such as phenolics and flavonoids, anthocyanidins, and tannins, which provide important health benefits such as antioxidant, anticancer, antiviral, anti-inflammatory activities, and an ability to inhibit human platelet aggregation activities (Dragsted *et al.*, 1993; Hanasaki *et al.*, 1994); Wang *et al.*, 1996); Fan *et al.*, 2008; Spada *et al.*, 2008; Mohsen & Ammar, 2009).

The classic examples of plant-derived compounds for drugs are vincristine, irinotecan, etoposide and paclitaxel. Whereas, actinomycin D, mitomycin C, bleomycin, doxorubicin and L-asparaginase are drugs originating from microbial sources, and citarabine is the first drug originating from a marine source. Some of these are in clinical use, others in clinical trials. Paclitaxel become one of the most effective drugs against breast and ovarian cancer and has been approved worldwide for the clinical treatment of cancer patients (Nobili *et al.*, 2009). Some studies have shown the positive correlation of the increased dietary intake of natural antioxidants with the reduced

coronary heart disease and cancer mortality, as well as with longer life expectancy (Halliwell, 2007; Rios *et al.*, 2009).

2.3 Natural Products and Their Economical Values

Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feedstock or raw materials for various scientific, technological, and commercial applications. Natural substances are employed, either directly or indirectly, by a large number of industries, and natural plant products (phytochemicals) figure prominently in several of these (Tyler *et al.*, 1981; Pryde *et al.*, 1981). Economically important plants serve as sources of industrial oils, tannins, saponins, natural rubber, gums, waxes, resins, dyes, pharmaceuticals, flavours and fragrances, pesticides and many specialty products.

Plant chemicals are often classified as either primary or secondary metabolites (Geissman & Crout, 1969; Balandrin & Klocke, 1988; Bell, 1980). Primary metabolites are substances widely distributed in nature, occurring in one form or another in virtually all organisms. As a general rule, primary metabolites obtained from higher plants for commercial used, they are mainly used as industrial raw materials, foods, or food additives and include products such as spices, vegetable oils, fatty acids (used for making soaps and detergents), and carbohydrates (for example, sucrose, starch, pectin, and cellulose). Such materials are generally valued at less than $1 to $2 per pound (about RM 5 to RM 10.50 per kg) and are readily available in large quantities in the market place. β-carotene is an expensive primary metabolite because its extraction, isolation, and purification are difficult.

Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Secondary metabolites have an ecological role; they are pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defences against microorganisms, insects and higher predators, and even other plants (allelochemics) (Harborne, 1972; Harborne, 2014; Kogan & Paxton, 1983; Putnam, 1983). Secondary metabolites are frequently accumulated by plants in smaller quantities than the primary metabolites. As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, fragrances, flavours and pesticides) are generally higher value-lower volume products than the primary metabolites. Thus, compared to primary metabolites (bulk chemicals), many secondary metabolites can be considered as specialty materials or fine chemicals.

Commercially useful plant secondary metabolites are nicotine, the pyrethrins and rotenone, which are used in limited quantities as pesticides and certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry (Tyler *et al.*, 1981). Compared to the relatively low cost of primary or bulk metabolites, secondary plant metabolites are often valued at several dollars to several thousand dollars per pound.

Lauraceae family is well known for its essential oil production. A high content of essential oils is found in many plants of the family that are important for spice or condiments (*Cinnamomum zeylanicum* Nees and *Cinnamomum cassia* Blume) (Vernin *et al.*, 1994; Bisset & Wichtl, 2001) and perfumery industries. *Cinnamomum zeylanicum* Nees and *Cinnamomum cassia* Blume also rich in essential oils (mainly

cinnamaldehyde and eugenol) which can inhibit microbial growth (Lee & Ahn, 1998; Friedman *et al.*, 2002; Burt, 2004; Ooi et *al.,* 2006). Avocados are important oil-rich fruit found in the members of this family and these are now planted in warm climates across the world to increase the production of oils. Moreover, many of these plants have very hard wood that is a source for timber worldwide (Ding *et al*., 1994; Weyerstahl *et al.*, 1994; Reynolds & Kite, 1995; Pino *et al.*, 2004). *Phoebe zhennan* S. K. Lee & F. N. Wei has been used as an ornamental plant in Chengdu Campagna (ChangTai *et al.*, 2009).

It is important to recall that the Industrial Revolution was made possible in part by the discovery and commercial development of the rubber tree, *Hevea brasiliensis.* However, since most species have never been described much less surveyed for chemical or biologically active constituents, it is reasonable to expect that new sources of commercially valuable materials remain to be discovered.

In 1965, Malaysia has a small pool of scientists to work on a large flora, as such will not be able to focus on a restricted area of research in natural products without compromising the freedom to do research. After year 2000 research on the natural products of Malaysian flora, Malaysia has not produced any compounds of pharmaceutical interest. Even in phytomedicinals, most of the raw materials are imported from India, China and Indonesia, even though the market is getting bigger. The involvement of government universities and a few research institutes that discuss their findings during the *Malaysian Natural Products Society Annual Meeting* is the solution to seek ways of improving the quality of their research. *The Malaysian Natural Products Society* was formed in 1994 and the committee are from their group of

researchers from the all of Malaysia government universities and have been going almost twenty years.

2.4 Lauraceae Family

2.4.1 General Information

Lauraceae family comprises a group of flowering plants. The members of the family are either trees or shrubs with aromatic oil glands, leaves alternate, rarely opposite and coriaceous. Stipules are absent in these plants. Flowers are small greenish or yellowish; bisexual or polygamous dioecious, regular, in cymes or racemes and sometimes mixed. During flowering, lauraceous species display thousands or millions of small (2-4mm diameter) light-colored flowers in panicles. The flowers are visited (and presumably pollinated) mainly by flies, bees and birds. Fruits are berry or drupe, often more or less enclosed by cup-like receptacle, which becomes fleshy. Lauraceous fruits make up 60-80% of all fruits eaten by bird species such as Three-wattled Bellbirds and Resplendent Quetzals. These birds may respond to annual variation in the availability of lauraceous fruits by migrating locally, by expending their diets to include previously ignored foods or unripe fruits, or by delaying breeding (Wheelwright, 1986). Family of Lauraceae contains over 3500 species from 55 genera (Rohwer, 1993) were distributed in tropical to subtropical areas, mainly in South America, Brazil and South-East Asia, (Simić *et al*., 2004). In world-wide, 213 species from 16 genera were recognised in Malaysia (Henry, 1949; Perry & Metzger, 1980). Lauraceae is one of the major families known as *Medang* or *Tejur* by Malays. About 1,300 are said to be medicinal plant from out of 12,000 species of flowering plants in Malaysia (Burkhill, 1935).

2.4.2 Botanical Classification of the Plant Study

The plant studied has botanical classification as follow;

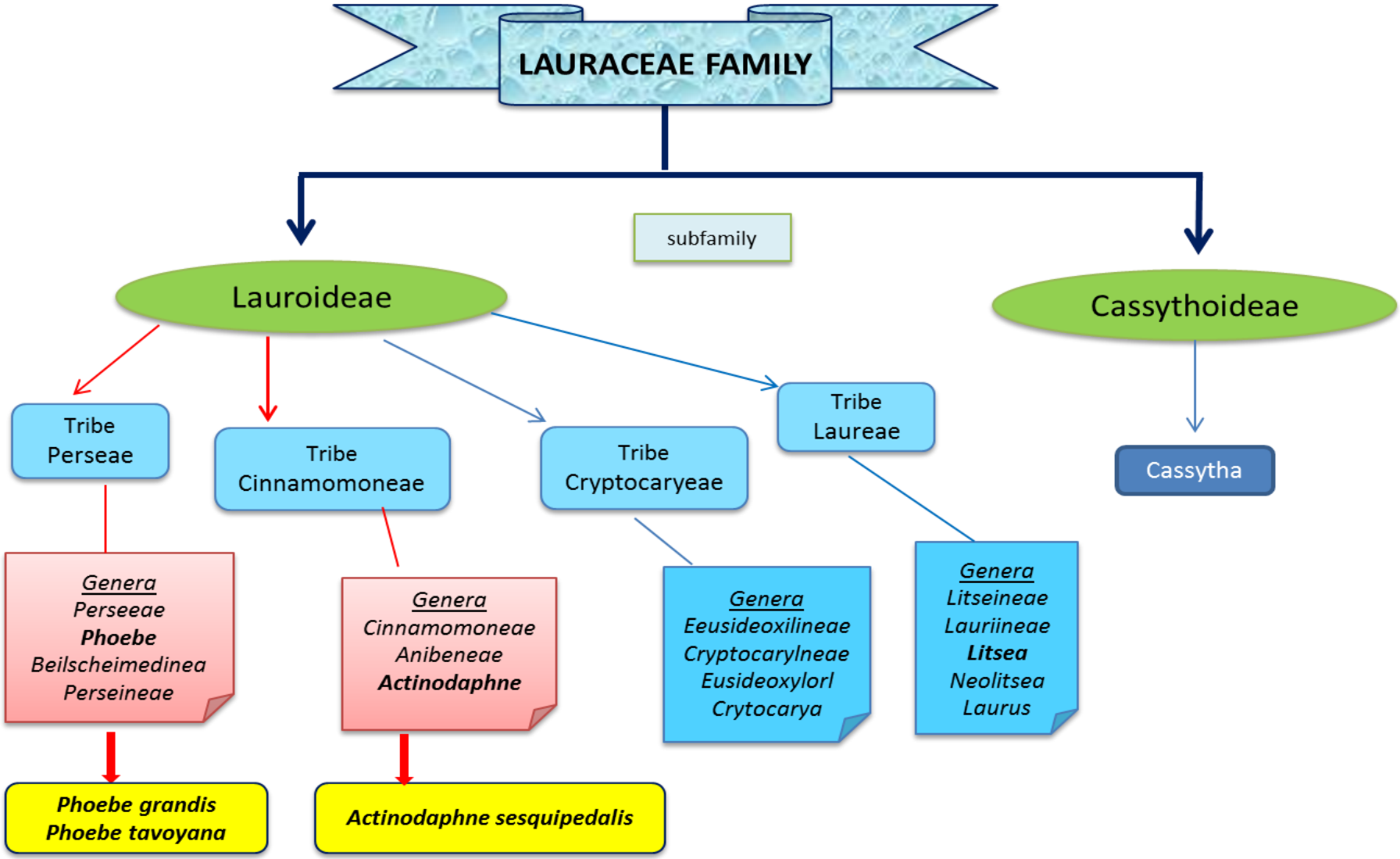
Kingdom	:	Plantae
Phylum	:	Magnoliophyta
Class	:	Magnoliatae
Sub class	:	Magnoliidae
Order	:	Laurales
Family	:	Lauraceae
Genus	:	*Phoebe*
	:	*Actinodaphne*

Classification of the species of the plant study from genus *Phoebe* and *Actinodaphne* are shown in the Scheme 2.1.

2.5 The Genus of *Phoebe*

2.5.1 General Information

The plants of the genus *Phoebe* are evergreen trees having alternate leaves, bisexual or polygamous flowers (pedicelled, in panicles; bracts dediduous; perianth lobes 6, slightly enlarge in fruits; stamen 9, outer 2 whorls introrse, inner whorl extrorse, 4^{th} whorl of staminodes; berry at the base of perianth lobes). *Phoebe* is a large genus of about 200 species, distributed in Indo-Malaysia, West Europe and Tropical America. Out of these species only 9 found in India (Gaur, 1999; Naithani, 1984).



Scheme 2.1: Classification of the studied plant from the Lauraceae family.

Genus *Phoebe* belongs to the family of Lauraceae of the *Perceae* tribe which is widely occurrence at tropical of Asia (Malaysia particularly) and America. It is most abundant in Borneo and Malaysian Peninsula from central Perak state to Malacca state. In Malaysia, the species are largely montane. Nonetheless, relatively few studies have been carried out. Several Malaysian species with their local names are listed as follow: *'**Medang Ketanah or Tanah' (P. grandis)**, 'Medang Asam'* (*P. cuneata blume),*, '*Medang Pasir'* (shore laurel), *'Medang Burung', 'Medang Keserai', Medang Merah', 'Medang Telur'*, *'Medang Kunyit', 'Medang Jambak,; 'Medang Inai';* and *'**Medang Rungkoi' (P. tavoyana* **Hook)**, are having a certain value as timber trees (Ridley, 2013; Corner, 1951). Also reported by Kochummen (1989) were several species belonging to the genus *Phoebe* such as *Phoebe attenuata, P. bourgeauviana, P. elliptica, P. lanceolata, P. macrophylla, P. Mexicana* and *P. sterculioides.* The *Phoebe* timber has no great commercial value although trees of commercial size were felled. They are suitable for decorative work such as furniture, panelling, interior finishing and cabinet-making. The heavier species could be utilized for medium construction under cover and plywood manufacture (Kochummen, 1989).

2.5.2 *Phoebe grandis* (Nees) Meer

Phoebe grandis belongs to the tribe of *Persea*. This timber tree is an evergreen with alternate leaves, often fascicle at the end of the twig. The tree is medium size to 12 m tall and 150 cm girth. The bark is pale to dark fawn-brown; uneven and rather rugged; and lenticellate. Inner bark is yellow or dark brown. Sapwood is yellow in colour. The leaves are frequently clustered at the end of the twigs. The stalk may have 1.2 - 5 cm long with blade leathery, obovate or oblanceolate. It is also variable and sometimes glaucous below. Young leaves pale yellowish and pinkish beneath. The colour of the

flower is normally yellowish brown. The fruits of this species may be bluish green, ellipsoid 1.5 x 0.75 cm seated on slightly enlarged lobed perianth. The species can be found in the lowland to the mountain forests throughout Malaysia, Java, Sumatra and Borneo. Figure 2.1 – Figure 2.4 below shows the characteristics of the *Phoebe grandis* which were recorded in the herbarium of University of Malaya, Kuala Lumpur.

Figure 2.1: Dried leaves and fruits of *Phoebe grandis* (Nees) Merr.

Figure 2.2: Fruits of *Phoebe grandis* elliptic or ovoid and bluish green. Flowers are normally yellowish brown in colour.

Figure 2.3: *Phoebe grandis* (Nees) Merr.- (KL 5540), the tree is about 20.0 m tall and 2.0 cm diameter.

Figure 2.4: *Phoebe grandis* (Nees) Merr.- (KL 5540), 21 cm diameter of bark (brown in colour) and inner bark pale brown in colour.

2.5.3 *Phoebe tavoyana* (Meissn.) Hk. F.

Phoebe Tavoyana (Meissn.) Hk. F. known locally as '*Medang Rungkoi'* is a small tree up to 14 meter tall. The leaves closely spirally arranged and appear whorled. The bark is grey brown, lenticellate, fissured whereas the inner bark is pale brown. The sapwood is yellow brown and the young twigs tomentose. It has densely hairy bud scales; stalk 1-2.5 cm long; blade thinly leathery, pubescent beneath when young, elliptic to lanceolate, 9-18 x 2.5-7 cm, apex with long tip; base cuneate; midrib raised above; secondary nerves 8-10 pairs, raised on both surfaces; tertiary nerves closely scalariform; faintly visible on both surfaces. The fruit ovoid, 1 x 0.6 cm, seated on lobed hairy perianth cup. Inflorescences from leaves axils, about 12 cm long with few branches, flowers with densely hairy perianth. Locally common found at lowland forests in Langkawi Island, Kedah, Perak, Kelantan, India, Burma, Indo-China, Thailand and Sumatra Indonesia. This species closely resembles to *P. lanceolata*, the hairy young twigs and perianth help to distinguish them (Kochummen, 1989). The characteristics of *Phoebe tavoyana* (Meissn.) Hk.F. (Lauraceae), shown in Figure 2.5 – Figure 2.8 had been recorded in the herbarium of University of Malaya, Kuala Lumpur.

Figure 2.5: *Phoebe tavoyana* leaves closely spirally arranged and appearing whorled.

Figure 2.6: Fruit of *Phoebe tavoyana* ovoid shape, 1 x 0.6 cm, seated on lobed hairy perianth cup.

Figure 2.7: Inflorescences of *Phoebe tavoyana* from leaf axils.

Figure 2.8: *Phoebe tavoyana* bark grey-brown in colour, lenticellate, fissured and inner bark pale brown in colour.

2.6 The Genus *Actinodaphne*

2.6.1 General Information

Actinodaphne is an Asian genus of the family Lauraceae. The name of the genus *Actinodaphne* come from Greek: *aktinos* means ray and *daphne* means laurel which alluding to the arrangement of leaves in star-shaped whorls. Laurel bay comprises a group of flowering plants within the order Laurels. It is a botanical genus of dioecious evergreen trees and shrubs with 140 species of the Lauraceae which commonly found in tropical and subtropical regions of Asia such as sourthen part of Korea, as well as in China (with 17 Chinese species), India and Japan (Kochummen, 1989). While, *Actinodaphne sesquipedalis* was widely found throughout Malaya (Rohwer, 1993; Van der Werff, 2001). They are trees of 3 to 25m tall, with leaves usually clustered or nearly verticillate. Many species are used locally for wood or medicine, i.e. the widely used wood of *A. nantoensis* (Hay.) Hay; and *A. mushanensis* (Hay.) Hay. used for architecture and furniture; whilst, the roots of *A. cupularis* (Hemsl.) Gamble and leaves of *A. pilosa* (Lour.) Merr. has the important medical properties (Kochummen, 1989).

In Malay Peninsula, the fruits of *A. sesquipedalis* are said to be stupefying, but they are eaten by birds. The leaves, stems, roots and barks are all considered to contain alkaloids.

2.6.2 *Actinodaphne sesquipedalis* Hook. F. var. glabra

Actinodaphne sesquipedalis Hook. F. known as "*Medang payung*" by the Malay people, which alluding to the arrangement of leaves in starshaped whorls (Kochummen, 1989). It was widely found throughout Malaya. This plant is native to Myanmar, Thailand, Peninsular Malaysia, and Borneo Island. Sub-canopy tree up to 24 m tall and 43 cm dbh (diameter at breast height). Stipules absent; Leaves in rosette, simple, penni-veined, hairy below, whitish below. Shoot bud protected by miniature leaves. Leaf under yellowish brown hairy and apex usually pointed. Figure 2.9 - Figure 2.11 below shows the characteristics of the *Actinodaphne sesquipedalis* Hook. F.

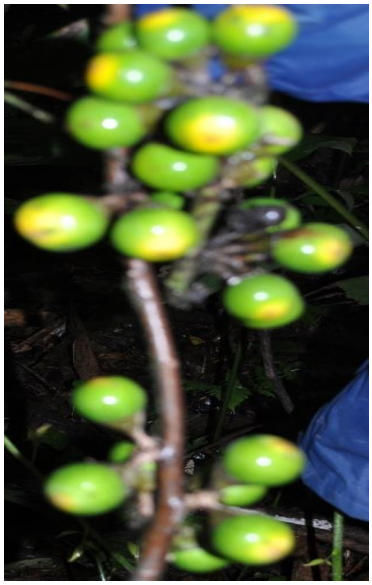


Figure 2.9: The fruit is drupe, berry, globose, subglobose or ellipsoid. The fruit is seated on the enlarged flat or concave perianth-tube.

Figure 2.10: The leaves texture is coriaceous with dark green above, glaucous or bluish-grey beneath, pinnately veined or rarely triplinerve and exstipulate.

Figure 2.11: Leaves of the small tree looks like umbrella shape.

2.7 Naturally occurring compounds isolated from *Phoebe* species

Phoebe species are rich in alkaloidal constituents (Bhakuni & Gupta, 1982; Castro *et al.*, 1986; Semwal *et al.*, 2009) and have been reported to contain the rare proaporphine-tryptamine dimers (Mukhtar *et al.*, 1997; Mukhtar *et al.*, 2004), aporphines (Mukhtar *et al.*, 1997; Stermitz & Castro C, 1983; Castro *et al.*, 1985; Chen *et al.*, 1997; Semwal *et al.*, 2008; Mukhtar *et al.*, 2009), proaporphine (Mukhtar *et al.*, 1997; Mukhtar *et al.*, 2009; Hufford & Morgan, 1976; Awang *et al.*, 2007) and oxoaporphines (Stermitz & Castro C, 1983; Mukhtar *et al.*, 2009; Awang *et al.*, 2007) compounds. Ten *Phoebe* species have been investigated for their chemical constituents and interestingly only two Malaysian species (*P. grandis* and *P. scortechinii*) produce proaporphine-tryptamine dimers. An interesting aporphine alkaloid named laurolitsine isolated from the stems of *P. formosana* (Hayata) has been used as a starting material to prepare bioactive phenanthrene alkaloids (Chiou *et al.*, 1998).

Table 2.1: Naturally occurring compounds from several *Phoebe* species.

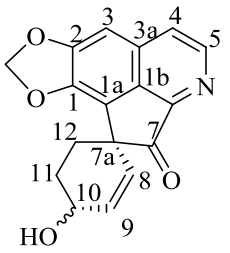
Species	Compounds	Type	References
Phoebe grandis (Malaysia)	Grandine A, **1** (-)-8,9-dihydrolinearisine, **2** Scortechiniine B, **3** Prooxocryptochine, **4** Boldine, **5** Norboldine, **6** Lauformine, **7** Scortechiniine A, **8**	Oxoproaporphine. Isoquinoline, Oxoproaporphine, Oxoproaporphine Aporphine, Aporphine, Proaporphine, Oxoproaporphine,	(Mukhtar *et al*., 2009)
Phoebe scortechinii (Malaysia)	(+) Scortechiniine A, **8** (+) Scortechiniine B, **3** (-) Hexahydromecambrine A, **9** (-) Norhexahydromecambrine A, **10** Norboldine, **6**	Oxoproaporphine Oxoproaporphine Proaporphine Proaporphine Aporphine,	(Mukhtar *et al*., 2008)
Phoebe lanceolata (India)	N-6/C-7 oxalyl-fused 2,9-dihydroxy-1,10-dimethoxy 6a,7-didehydroaporphine, **11** N-6/C-7 oxalyl-fused 1,2,9,10-tetramethoxy 6a,7-didehydroaporphine, **12**	Oxalyl-fused didehydroaporphine Oxalyl-fused didehydroaporphine	(Semwal *et al*., 2008)
Phoebe grandis (Nees) Merr. (Malaysia)	Phoebegrandine D **13** Phoebegrandine E **14**	Proaporphine-tryptamine dimer, Indoloquinolizidine	(Awang *et al*., 2006)
Phoebe grandis (Nees) Merr. (Malaysia)	Phoebegrandine C, **15** Phoebegrandine A, **16** Phoebegrandine B, **17** Tetrahydroglaziovine, **18**	Proaporphine-tryptamine dimer, Proaporphine-tryptamine dimer, Proaporphine-tryptamine dimer, Proaporphine	(Mukhtar *et al*., 2004)

Table 2.1: (continued) Naturally occurring compounds from several *Phoebe* species.

Species	Compounds	Type	References
Phoebe lanceolata (Malaysia)	Liriodenine, **19** Roemerine, **20** Boldine, **5** Norboldine, **6** Laurotetanine, **21** (-)-Sebiferine, **22** Asimilobine, **23**	Oxoaporphine, Aporphine Aporphine Aporphine Aporphine Morphinandienone (First alkaloid reported present in the bark of *Phoebe* species) Aporphine	(Mukhtar, 2003)
Phoebe grandis (Malaysia) Bark *Phoebe grandis* (Malaysia) leaves	Boldine, **5** Norboldine, **6** Laurotetanine, **21** Lindcarpine, **24** Phoebegrandine A, **16** Phoebegrandine B, **17**	Aporphine, Aporphine, Aporphine Aporphine Proaporphine-tryptamine dimer Proaporphine-tryptamine dimer	(Mukhtar *et al*., 1997)
Phoebe Chekiangensis	5-Hydroxy-indoline, **25** Tyramine, **26** N-norarmepavine, **27** Glycosylated tetrahydroisoquinoline SCH 71450, **28**		(Hegde *et al*., 1997)
Phoebe farmosana (Taiwan)	6,7-oxalyl-fused dehydrorolitsine (Laurodionine, **29)**	Oxalyl-fused dehydroaporphine,	(Chen *et al*., 1997)
Phoebe clemensii (New Guinea)	Isocorydine, **30** *N*-methyllindcarpine, **31** Laurolitsine @ norboldine, **6**	Aporphine Aporphine Aporphine	(Stermitz & Castro C, 1983)
Phoebe farmosana (Taiwan)	Roemerine, **20** Laurolitsine @ norboldine, **6** Ushinsunine, **32** Liriodenine, **19**	Aporphine, Aporphine Aporphine Oxoaporphine	(Stermitz & Castro C, 1983)

Table 2.1: (continued) Naturally occurring compounds from several *Phoebe* species.

Species	Compounds	Type	References
Phoebe molicella	Norpreocoteine, **33** Norpurpureine, **34** Preocoteine, **35**	Pentasubstituted Aporphine Aporphine Aporphine	(Stermitz & Castro C, 1983)
Phoebe farmosana	Lauformine, **7** *N*-methyllauformine, **36**	Proaporphine Proaporphine	(Lu & Tsai, 1984)
Phoebe valeriana	Phoebine, **37** Norphoebine, **38** Oxophoebine, **39**	Aporphine Aporphine Oxoaporphine	(Castro *et al*., 1986)



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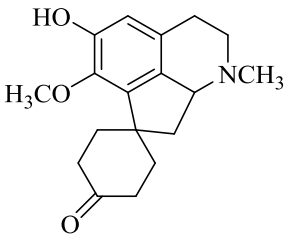

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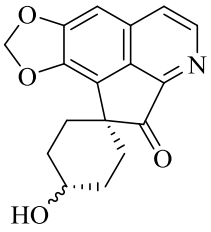

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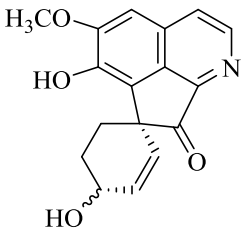

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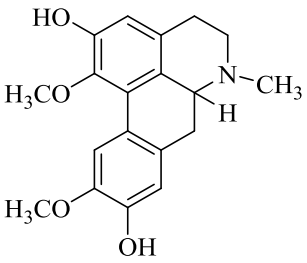

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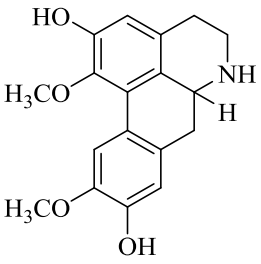

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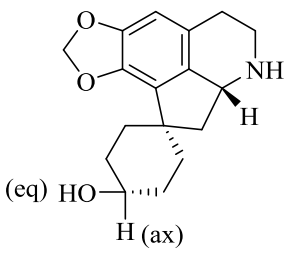

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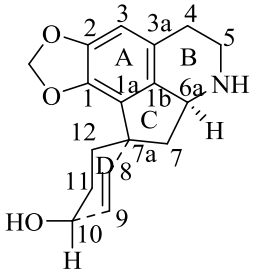

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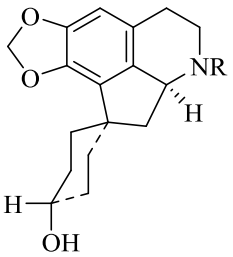
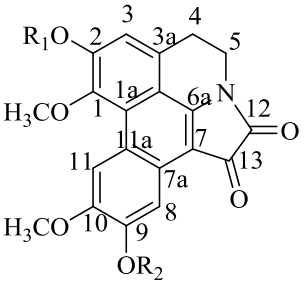
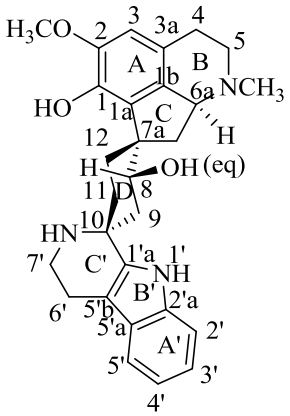


9: R=CH3
10: R=H



	R1	R2
11:	H	H
12:	CH3	CH3



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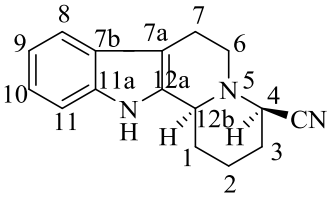

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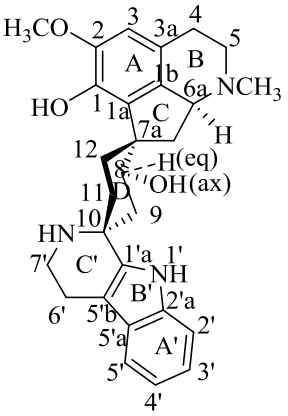

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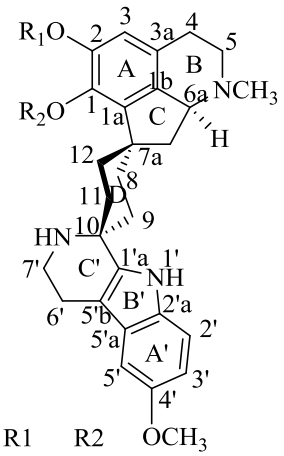
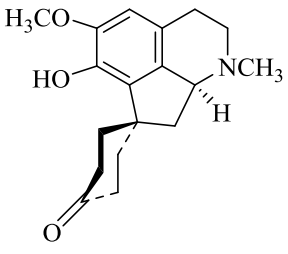


	R1	R2
16:	CH3	H
17:	H	CH3



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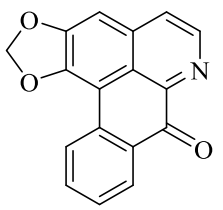

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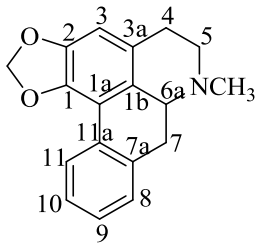

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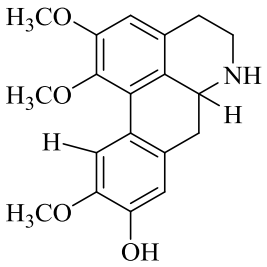

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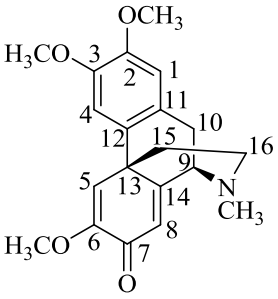

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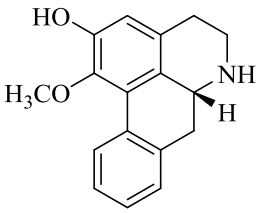

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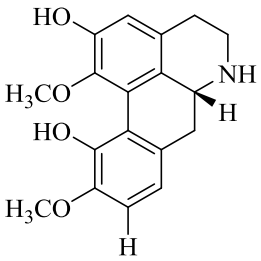

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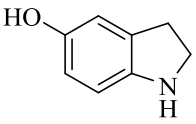

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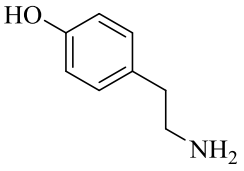

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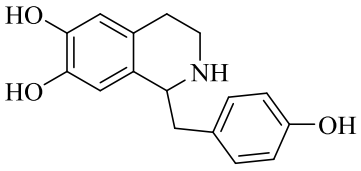

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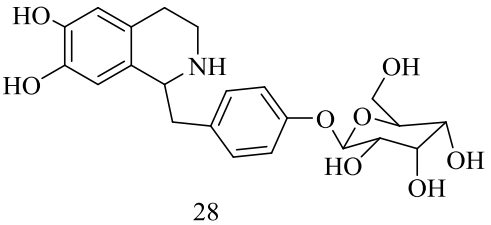

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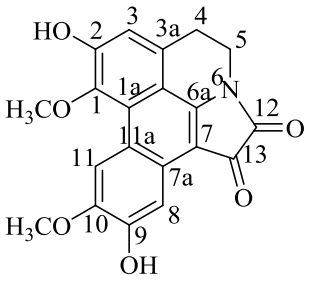

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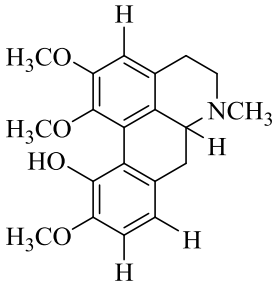

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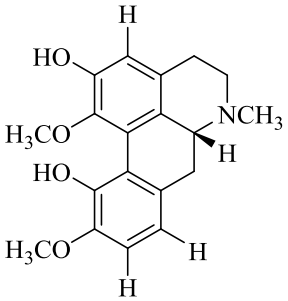

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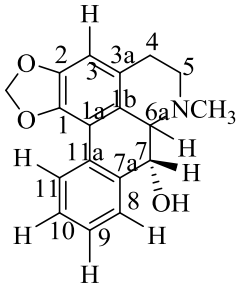

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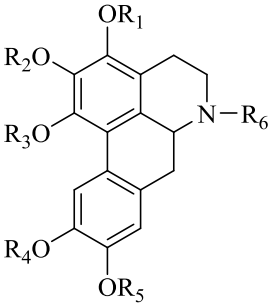
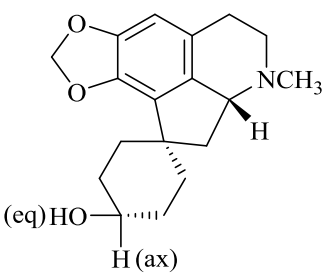


33: R1=R2=R4=R5=CH3; R3=R6=H
34: R1=R2=R3=R4=R5=CH3; R6=H
35: R1=R2=R4=R5=R6=CH3; R3=H



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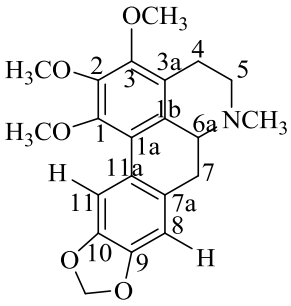

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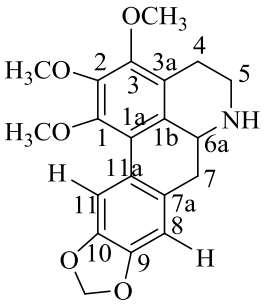

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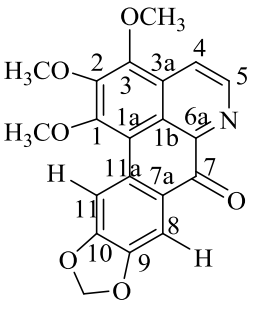

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2.8 Naturally occurring compounds isolated from *Actinodaphne* species

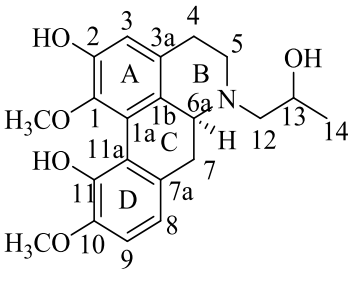
Actinodaphne plants of the family Lauraceae have been reported to produce isoquinoline alkaloids (aporphines, oxoaporphines) and lactones (Uprety *et al.*, 1972; Kim *et al*., 2002). Aporphine alkaloids broadly exist in nature and have obvious biological activity with the great content.

Table 2.2: Naturally occurring compounds from several *Actinodaphne* species.

Species	Compounds	Type	References
Actinodaphne pruinosa Nees (bark)	(+)-N-(2-hydroxypropyl)lindcarpine, **40** boldine, **5** Laurolitsine @ norboldine, **6** Lindcarpine, **24** and methyllindcarpine, **31**	Aporphine Aporphine Aporphine Aporphine Aporphine	(Rachmatiah *et al*., 2009)
Actinodaphne lancifolia (stem)	Actinolide A, **41** Actinolide B, **42** Isolancifolide, **43** Lancifolide, **44** Secoisolancifolide, **45** Litsenolide C_1, **46** Litsenolide C_2, **47** (±)-syringaresinol, **48** (±)-de-4'-o-methylmagnolin, **49** Lyoniresinol, **50**	Lacton Lacton Lacton Lacton Lacton Lacton Lacton Lignan Lignan Lignan	(Kim *et al*., 2002)
Actinodaphne speciosa Nees	Laurotetanine, **21** N-methyllaurotetanine, **51**	Aporphine Aporphine	(Laili Din *et al*., 1994)
Actinodaphne pruinosa Nees	Laurolitsine @ norboldine, **6**	Aporphine	(Laili Din *et al*., 1994)
Actinodaphne sespequipedalis Hook. F	Dicentrine, **52**	Aporphine	(Laili Din *et al*., 1994)

Table 2.2: (continued) Naturally occurring compounds from several *Actinodaphne* species.

Species	Compounds	Type	References
Actinodaphne nitida Teschn.	Boldine, **5** Laurolitsine, **6**	Aporphine Aporphine	(Johns *et al.*, 1969)
Actinodaphne hookeri Meissn	Actinodaphnine, **53**	Aporphine	(Johns *et al.*, 1969)



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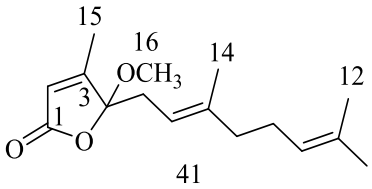

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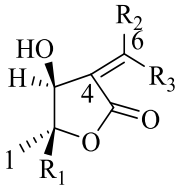
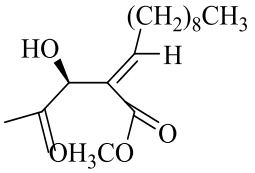


	R_1	R_2	R_3
42:	OCH_3	H	$(CH_2)_8CH_3$
46:	H	H	$(CH_2)_8CH_3$
47:	H	$(CH_2)_8CH_3$	H



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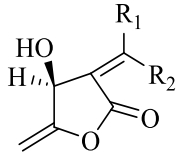
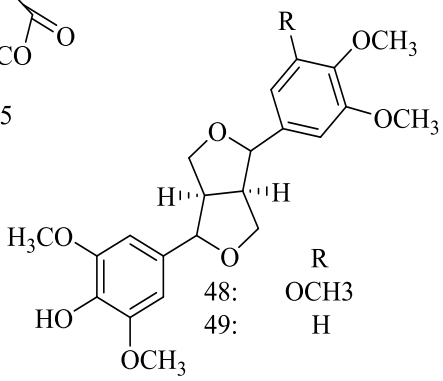
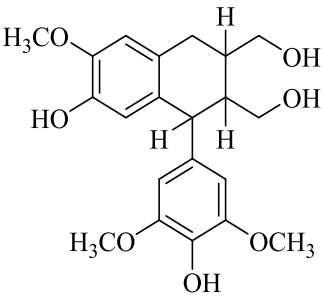


	R_1	R_2
43:	$(CH_2)_8CH_3$	H
44:	H	$(CH_2)_8CH_3$



	R
48:	OCH3
49:	H



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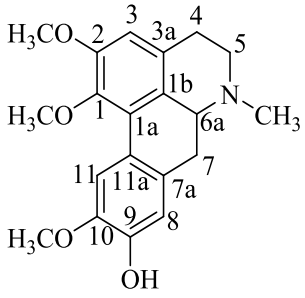

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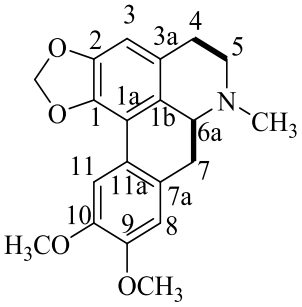

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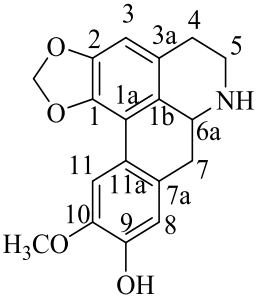

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2.9 Bioactivities.

Lauraceae family become important species since plants were found to exhibit useful biological activities such as antispasmodic, antipyretic, antitumour, anticonvulsant and antiviral. They contained interesting classes of natural products, such as alkaloids, monoterpenes and sesquiterpenes, triterpenes and sterols, 2-pyrones, flavonoids, benzophenones and arylpropanoids (including lignans and neolignans). In addition, compounds from the medicinal plants of this family have been found to show antioxidant activities (Bruni *et al.*, 2004; Hwang *et al.*, 2005; Omar *et al.*, 2013; Elya *et al.*, 2014) which can promote antiproliferation of the human cancer cells (Wang *et al.*, 2008).

2.9.1 Previous reports on biologically active components of *Phoebe* species.

Beside the Malay Peninsula, *Phoebe* species also were reported in China, Indonesia, Indochina, Japan and The Philippines (Perry & Metzger, 1980) to be useful for treatment of several diseases thanks to their antidiabetic, antibacterial, antifungal, antitumor and antiplasmodial activities (Semwal *et al*., 2008; Omar *et al.*, 2013; Elya *et al.*, 2014). Its traditional uses inspired us to carry out test for free radical scavenging and antioxidant activity.

Aqueous extract of berries from *Phoebe lanceolata* is an important remedy for wounds. The extracts have been used to cure tuberculosis, asthma, diabetes, psycho-disorders, dysentery and fever (Gaur, 1999). Whereas ethanolic extracts of *P. lanceolata* stem bark were evaluated for their antibacterial activity against five bacterial species: *Staphylococcus aureus* (along with ten hospital-derived strains), *Staphylococcus*

mutans, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* with MIC range of 50 – 100 µg/mL (Semwal *et al*., 2009).

A proaporphine alkaloid which occurs in *P. grandis* is used as a precursor in the synthesis of aporphines and proaporphine-tryptamines alkaloids (Mukhtar *et al.,* 2009). These alkaloids are known for their unique pharmacological activities, as demonstrated by liriodenine which shows antitumor, antibacterial, and antifungal activities (Awang *et al.*, 2007). Biological screening on the crude alkaloidal extract of the leaves of *Phoebe grandis* for antiplasmodial activity has shown positive results too: $IC_{50} < 8$ µgmL^{-1} (Awang *et al*., 2006).

It was reported that declinine is a new alkaloid from *Phoebe declinata* Nees exhibited a good antioxidant agent with IC_{50} 11.8 µg/mL which is compared to boldine as alkaloid standard with IC_{50} 5.8 µg/mL by the DPPH method (Elya *et al.,* 2014). The CH_2Cl_2 extract of the bark of *Phoebe scortechinii* has shown antiplasmodial activity to resistant strain *P. falsiparum*, Gombak A (IC_{50} = 0.1042 µgmL^{-1}) and to sensitive strain *P. falsifarum*, D10 (IC_{50} = 0.1527 µgmL^{-1}) (Mukhtar *et al.,* 2008). Results from this preliminary investigation suggest that these Lauraceae tree species may have a great potential for further development as cancer chemoprevention agents or food supplements for promoting human health (Lin *et al.*, 2007).

2.9.2 Previous reports on biologically active components of *Actinodaphne* species

Several species of the genus *Actinodaphne* Nees are occasionally used and some species are poisonous. In China, remedy for trauma made from the bruised leaves of *A. cupularis* (Hemsl.) Gamble. People in Indo-China made tea from leaves of *A. pilosa*

(Lour.) Merr. as a drink to treat stomach ache but *A. perakensis* (Gamble) Kosterm is a poisonous species. In Indonesia, the crushed or ground young leaves of *A. moluccana* Blume are smeared on boils and also on wounds.

Meanwhile, the wood of an unnamed collection of *Actinodaphne* Nees, after charring is used as an astringent in New Guinea (Perry & Metzger, 1980). The root of *A. lancifolia* (Sieb. et Zucc.) Meissn. Var, *sinensis* Allen, is a traditional Chinese medicine used for the treatment of stomach ache, arthritis, overexertion and edema (Kim *et al.*, 2002).

Actinodaphne plant species provide various biological active compounds such as dicentrine (**52**), liriodenine (**19**), (+)-N-(2-hydroxypropyl)lindcarpine (**40**), boldine (**5**), norboldine (**6**), lindcarpine (**24**) and methyllindcarpine (**31**); and are of phytopharmaceutical importance.

Liriodenine (**19**), an oxoaporphine, was reported to have antitumor, antifungal and antibacterial activities (Leboeuf *et al.*, 1980). In addition, dicentrine (**52**), an aporphine was known to have cytotoxic activity against P-388 murine cells (Likhitwitayawuid *et al*., 1993). (+)-N-(2-hydroxypropyl)lindcarpine (**40**) from *Actinodaphne pruinosa* Nees exhibited cytotoxic activity against P-388 murine leukemia cells with an IC_{50} value of 3.9µg/ml (Rachmatiah *et al*., 2009).

2.10 Cancer and its Treatment

Cancer is the most common and fatal disease and accounted for 7.6 million deaths (about 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 (Tayarani-Najaran & Emami, 2011). Breast

cancer is one of the main life threatening diseases that a woman may have to face during her lifetime and is the most widespread (Parkin & Fernández, 2006). While incidence rates have historically been higher in the developed countries, there has been a recent sharp increase in incidence and mortality in the developing countries (Porter, 2009). The increasing incidence of breast neoplasia reported over the last a few decades has led to development of new anticancer drugs, drug combinations, and natural products.

In the Western World, ovarian cancer is the number one gynaecologic killer and is the fourth most common malignancy in women (Sugarbaker, 2009). Approximately 1 in 75 women in the developed countries will be affected by ovarian cancer with at least two thirds of women presenting late with the disease because of non-specificity symptoms and often a delay in the diagnosis (Jasen, 2009). In some countries, there is a lack of effective cervical cancer screening programs. The incidence of cervical cancer has not reduced significantly during the past three decades (Sankaranarayanan *et al.*, 2009).

Herbal medicines have a vital role to play in the prevention and treatment of cancer. About 60% of anticancer drugs used nowadays were obtained from natural sources (Itharat, 2009). Out of total 250,000 plant species existing on Earth approximately one thousand have known anticancer potential (Abu-Dahab & Afifi, 2007). A large number of plant species have been screened through bioassays in the search for novel plant-based anticancer drugs. With advanced knowledge of molecular science and refinement in isolation and structure elucidation techniques, various anticancer compounds have been identified, which execute their therapeutic effect by inhibiting cancer-activating enzymes and hormones, stimulating DNA repair mechanisms, promoting production of protective enzymes, inducing antioxidant action and enhancing immunity of the body (Sakarkar & Deshmukh, 2011).

Two of the common anti-cancer drugs which are derived from the plants are vincristine and vinblastine. Vincristine is used in the chemotherapeutic regimen for Hodgkin's lymphoma while vinblastine is used for childhood leukemia (Johnson *et al*., 1963). The search for improved cytotoxic agents as part of a programme for discovering modern drugs to fight cancer is on-going.

Cancer chemopreventive activity of resveratrol, a natural product derived from grapes skin, red wine, berries, peanuts, and medicinal plants, such as Japanese knotweed (*Polygonum cuspidatum*) and other food products was found to act as antioxidant and antimutagen and to induced phase II drug-metabolizing enzymes (anti-initiation activity); it mediates anti-inflammatory effects and inhibits cyclooxygenase and hydroperoxidase functions (antipromotion activity). Furthermore it induces human promyelocytic leukemia cell differentiation (antiprogression activity) and inhibits the development of preneoplastic lessions in carcinogen-treated mouse mammary glands in culture and inhibits tumorigenesis in a mouse skin cancer model. These data suggest that resveratrol, a common constituent of the human diet, merits investigation as a potential cancer chemopreventive agent in humans (Jang *et al.,* 1997; Pollack & Crandall, 2013).

2.11 Free radicals

According to Packer and Colman (1999), free radicals are highly reactive molecules with an odd number of electrons. Free radical reactions occur in the human body and food systems. Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals (Gutteridge & Halliwell, 1993). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism

(Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide ($O_2^{\cdot -}$) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO-), and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide (NO) and peroxynitrite anion ($ONOO^{\cdot -}$). These reactive species can react with biomolecules, causing cellular injury and death. This may lead to the development of chronic diseases such as cancers and those that involve the cardio- and cerebrovascular systems. In treatment of these diseases, antioxidant therapy has achieved considerable importance. The consumption of fruits and vegetables containing antioxidants has been found to offer protection against these diseases. Dietary antioxidants can augment cellular defences and help to prevent oxidative damage to cellular components (Halliwell, 1989).

2.12 Bioassay

2.12.1 MTT Cytotoxic Assay

Many biological assays require the measurement of surviving, proliferating and/or activation of cells. The requirements for a sensitive, quantitative, reliable and automated method have led to the development of radioactive assays based either on the capacity of cells to incorporate a radioactive substrate ([^{3}H]-thymidine, [^{3}H]-uridine) or on their capacity to release a radioactive marker (^{51}Cr, [^{3}H]-thymidine, ^{125}I-deoxy-uridine). Mosman in 1983 introduced the alternative method to measure viable cells to transforms the MTT tetrazolium salt into MTT formazan (Gerlier & Thomasset, 1986). Among the methods widely developed is colorimetric assay.

The metabolic activity of viable cells is one of parameter used as basis for colorimetric assays. Ideally colorimetric assay for living cells should utilise a colourless substrate that is modified to a coloured product by any living cell, but not by dead cells or tissue

culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells. A rapid colorimetric assay, based on tetrazolium salt MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenytetrazolium bromide) that is measured only in living cells. The result of this reaction can be read on a scanning multiwall spectrophotometer (ELISA reader), using a test wavelength of 570 nm and a reference wavelength of 630 nm. This assay is capable of detecting very small number of living cells (eg. 200). The actual cells do not absorb significantly, even at a concentration of 1 x 10^6 cells/mL (Mosmann, 1983). This method was then improved by several researchers (Denizot & Lang, 1986; Gerlier & Thomasset, 1986; Twentyman & Luscombe, 1987).

MTT, a pale yellow substrate is reduced to a dark blue formazan by the "succinate-tetrazolium reductase" system (Figure 2.12), which belongs to the mitochondrial respiratory chain and is active only in viable cells. The MTT soluble formazan reaction product was only partially soluble in the medium, and so an alcohol was used to dissolve the formazan and produce a homogeneous coloured solution. The optical density of this solution can be quantified spectrophotometrically at 570 nm.

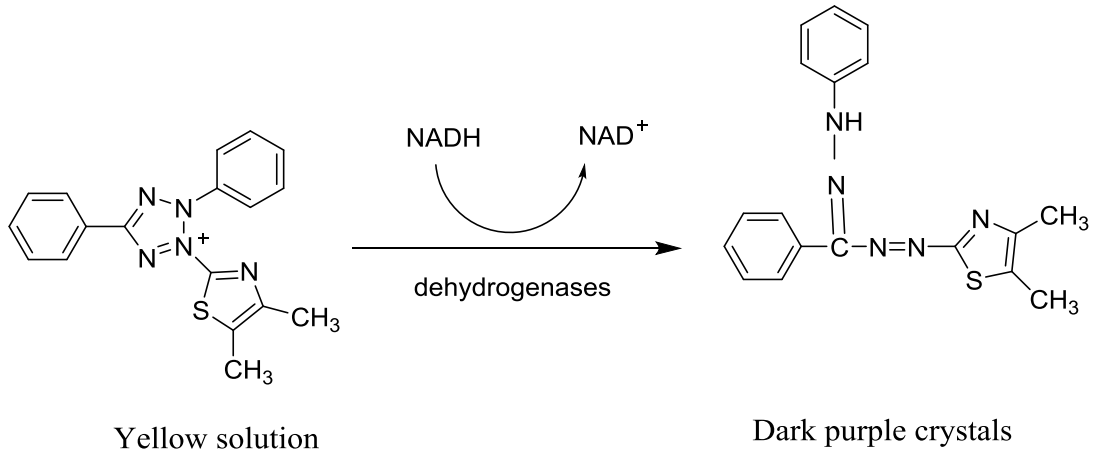


Figure 2.12: Molecular structure of MTT and formazan.

The colorimetric methods have several advantages over radioactive methods. They are less time consuming, cheaper, and the use of multiwell scanning spectrometers (ELISA readers) permits the processing of large number of samples (Gerlier & Thomasset, 1986).

2.12.2 Antioxidants assay

Antioxidant compounds in food play an important role as a health-protecting factor. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease (Prajapati *et al*., 2009; Wong *et al*., 2006). Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin A, vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognised as having the potential to reduce disease risk.

Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

Synthetic antioxidants, such as propyl gallate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylhydroquinone (TBHQ), have been added to foodstuffs to control lipid oxidation in foods. Although these synthetic antioxidants are efficient and relatively cheap, there are some disadvantages, because they are suspected of having some toxic properties. Therefore search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones.

Plants with the potent antioxidants activity are now receiving a special attention (Ghosal *et al*., 2003; Wenli *et al*., 2004) to be investigated in the recent scientific developments throughout the world. Some of plants have no side effects and economic viability (Auddy *et al*., 2003). A large number of phenolic compounds (Ogiwara *et al*., 2001), tannins (Okuda, 2005), flavonoids (Fauré, *et al*., 1990) present in vegetable foods, such as fruits and nuts, have been reported to possess good antioxidant properties, free radical scavenging abilities, anti-inflammatory, anticarcinogenic, antiatherosclerotic, antitumor, antimutagenic, anti-bacterial or antiviral activities to a greater or lesser extent (Ali et al., 2005; Miller, 1996). Moreover, aromatic plants contained with the essential oils showed very good potential antioxidative effects for the preservation of the foods from the toxic effects of the oxidants (Zygadlo *et al*., 1995; Maestri *et al*., 1996; Maestri *et al.*, 1997; Tepe *et al*., 2004).

Numerous studies have demonstrated that the antioxidant activity measurement depend substantially on the test system used (Janaszewska & Bartosz, 2002; Bauzaite *et al*., 2003) and it is recommend that any conclusions be based on at least two different test systems (Frankel *et al*., 1994; Chu *et al*., 2000; Koleva *et al*., 2002; Nuutila *et al*., 2003; Moon & Shibamoto, 2009) due to the complex nature of phytochemicals.

The BCB (β-carotene bleaching method) employs an emulsified lipid, which introduces a number of variables influencing antioxidant activity of samples. The BCB method can be helpful especially for investigations of lipophilic antioxidants and it is appropriate for the investigation of the antioxidant activity of essential oils. On the other hand if polar compounds as ascorbic acid, rosmarinic acid, etc, are tested only by the BCB method, they would be considered as weak antioxidants (Kulisic *et al*., 2004).

2.12.2.1 DPPH free radical scavenging assay

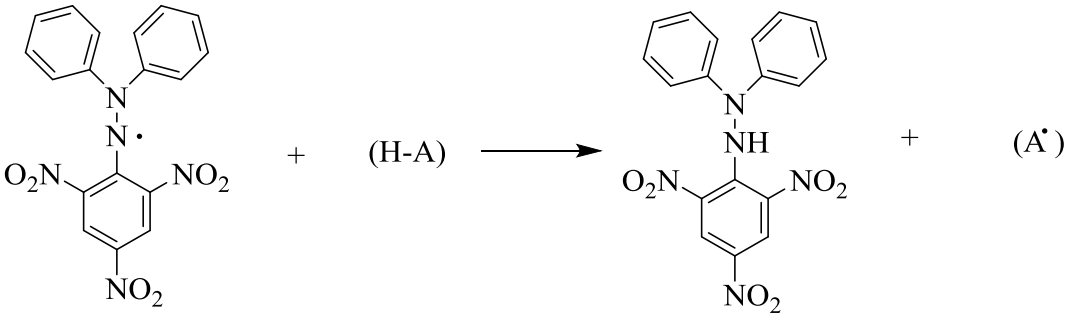
The DPPH method is faster than BCB method and it can be helpful in investigation of novel antioxidants for a rapid estimation and preliminary information of radical scavenging abilities. The method is sensitive and requires small sample amounts (Blois, 1958). TBA method is also sensitive and achieves reproducible results. This method is preferable in order to obtain useful data in an environment similar to the real-life situation. Both methods, DPPH and TBA, similarly allow testing of both lipophilic and hydrophilic compounds (Kulisic *et al*., 2004), (Koleva *et al*., 2002). However, the antioxidant power depends on the chosen method, on the concentration and on the nature and physicochemical properties of studied antioxidants (Koleva *et al*., 2002).

DPPH assay is one of the most widely used methods for the screening of antioxidant activity of the plant extracts (Gupta *et al*., 2009). DPPH is a relatively stable free radical scavenger which converts the unpaired electrons to paired ones by hydrogen proton donation (Wintola & Afolayan, 2011). The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 517nm. DPPH is very popular for the study of natural antioxidants (Villano *et al*., 2006). The PubMed database shows that this radical has been employed in more than 850 studies since 1969.

2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical and ferric reducing antioxidant power (FRAP) method have been employed in this study for detection of the antioxidant activity of the extracts and active compounds. The spectrophotometric technique employs the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) showing a characteristic UV–vis spectrum with a maximum absorbance close to 517 nm in methanol (Que *et al*., 2006; Villano *et al*., 2006; Katalinić *et al*., 2004; Katalinic *et al*., 2005). Antioxidant compounds can donate hydrogen atom to DPPH free radicals thus quenching the radical. Colour of DPPH solution changed from purple to pale yellow. Exceeded of DPPH free radical from the reaction can absorb visible light. Therefore the absorbance can be measured by spectroscopic method at 518 nm (Milardović *et al*., 2006).

DPPH assay: 2,2-diphenyl-1-picrylhydrazyl (Oktay *et al*., 2003). Principle:

The scavenging reaction between (DPPH.) and an antioxidant (H-A) can be written as:



Purple Yellow

$$(DPPH\bullet) + (H\text{-}A) \rightarrow DPPH-H + (A\bullet)$$

Antioxidants react with DPPH• , which is a stable free radical and is reduced to the DPPHH and as consequence the absorbance is decreased consistent with the change from DPPH• radical to the DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Oktay *et al*., 2003).

2.12.2.2 Ferric reducing antioxidant power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to ferrous form (Fe^{2+}-TPTZ). It will form an intense blue Fe^{2+}-TPTZ complex with an absorbance maximum at 593 nm (Benzie & Strain, 1996). Increasing absorbance indicates an increase in reductive ability.

2.12.3 Antibacterials

Over the last decade, antibiotics which were considered to be miracle drugs have been losing their effectiveness as pathogens evolve resistance against them (Saleem *et al*., 2010). Bacterial resistance to antibiotics is a serious health problem and has been identified for all classes of antibiotics available (Wood *et al*., 1996). The resistance of gram-positive pathogens to antibiotics is a great concern because these bacteria pose a continuous and serious threat to public health and lead to a high degree of mortality (Sefton, 2002).

Antibacterial resistance continues to grow quickly among key pathogens such as *Staphylococcus epidermidis* (a Gram-positive bacterium), which is a part of human skin flora and also can be found in the mucous membranes and in animals (Schleifer & Kloos, 1975), *Staphylococcus aureus* a gram-positive pathogen of significance in public health, is currently the most frequent by cause of nosocomial bacteremia and

skin/wound infections and the second most frequent cause of nosocomial lower respiratory infections (Wang *et al*., 2012), and it is estimated that 20% of the human population are long-term carriers of *S. aureus* (Kluytmans *et al*., 1997; Cole *et al*., 2001), *Bacillus subtilis* (Gram-positive bacterium) commonly found in soil, and more evidence suggests that *B. subtilis* is a normal gut commensal in humans (Hong *et al*., 2009), *Pasteurella multocida*, a Gram-negative bacterium, causes a range of diseases in mammals and birds, including fowl cholera in poultry (Boyce & Adler, 2000; Harper *et al*., 2004) and *Enterobacter cloacae*, a Gram-negative bacterium, is commonly found in infections among burn victims, immune compromised patients, and patients with malignancies (Musil *et al*., 2010). While, *Escherichia coli,* a Gram-negative bacterium, has been responsible for different outbreaks of diseases associated with chronic diarrhoea and the dysfunctioning of the kidney which in some cases can be lethal (Mayer & Wanke, 1995).

In 2004, about 2 million people acquire bacterial infections in US hospitals, 70% of cases involved strains that are resistant to at least one drug (America, 2004). A major cause for concern in the UK is methicillin-resistant *Staphylococcus aureus* (MRSA) (Adcock, 2002).

This concern has encouraged researchers to search the efficient methods for preventing and curing the harmful effects of microbes in a more eco-friendly manner. Biocides derived from plants are safer, more effective and environment-friendly. They are rich in bioactive secondary metabolites such as alkaloids, flavonoids, terpenes, coumarins and saponins. Substantial investment and research in the field of anti-infectives is now desperately needed if a public health crisis is to be averted. Several investigators have

demonstrated potential of some plants as possible sources of antimicrobial compounds (Huang *et al*., 2010; Motiejūnaite & Peciulyte, 2003).

In our efforts to screen plants for antimicrobial activity, we demonstrated the potential of extracts of *P. grandis, P. tavoyana and A. sesquipedalis* as an antibacterial agent using *Bacillus subtilis* (gram-positive), *Staphylococcus aureus* S1434 (gram-positive), *Staphylococcus epidermidis* (gram-positive), *Escherichia coli* (gram-negative), *Salmonella typhi* (gram-negative) and Methicillin resistant *Staphylococcus aureus* (MRSA) (gram-positive).

In general, several methods have been employed in plant extracts and individual compound screening of antibacterial activity. According to Rios *et al*., (1988) and Hamburger & Hostettmann, (1991), antibacterial assays can be categorised into three groups, namely diffusion, dilution and bioautographic methods. The principles for each method as described by (Rios *et al*., 1988) are as follows:

a) Diffusion methods

   This is a technique which does not require homogeneous dispersion in water. The principal diffusion method is the agar-overlay method using a disk, hole or cylinder as a reservoir. The reservoir containing the sample to be tested is brought into contact with an inoculated medium. Then, after incubation, the diameter of the clear zone around the reservoir (inhibition diameter) is measured. This method was originally designed to monitor the amount of antibiotic substances in crude extracts.

In this technique, filter paper discs are saturated with the sample solution and placed on the surface of agar immediately after inoculation with the organism tested. The hole-plate assay method depends upon the diffusion of the antibiotic from a vertical hole through the solidified agar layer of a petri dish or plate to such an extent that growth of the added microorganism is prevented entirely in a circular area or zone around the hole containing a sample solution. The cylinder method is similar to the hole-plate method. Stainless steel or porcelain cylinders are used for the assays. After incubation, the cylinders are removed, and the average diameter of each zone of growth inhibition is measured and recorded.

The advantages of these methods are the small size of the sample used in the screening and the possibility of testing five or six compounds against a single microorganism. The diffusion methods suit for preliminary screening of pure substances (alkaloids, flavonoids, terpenoids, etc.).

b) Dilution methods

The techniques required a homogenous dispersion of the sample in water. They are used to determine the minimum inhibition concentration (MIC) values of extracts, pure compounds or essential oil. The liquid dilution method, turbidity is taken as indication of bacterial density. When no growth takes place, the medium remains clear (sample active) and when there is a growth, the medium becomes turbid (sample inactive). The grade of inhibition is related to the turbidity of the medium and measured by spectrophotometry.

c) Bioautographic methods

Bioautography is the most important detection method for new or unidentified antimicrobial compounds. It is based on the biological effects of the substances under study. This method is based on the so-called agar-diffusion technique, whereby the separated compounds are transferred from the chromatographic layer (thin layer chromatography or paper chromatography) to the inoculated agar plate for 15-30 minutes to let the sample tested diffuse into the agar layer and the sheets are removed. The plates are incubated at appropriate temperature until a thin film of the growing microorganism is visible on the surface. Inhibition zone are visualised by dehydrogenase-activity-detecting reagents.

2.12.4 Antiplasmodial Assay

Plasmodium falciparum is a major parasitic infection disease in the world and continues to cause morbidity and mortality on a large scale in tropical countries and undermining development in the poorest countries of the world [(WHO), World Malaria Report 2014]. *P. falciparum* is most prevalent on the African continent, and is responsible for most deaths from malaria [(WHO), World Malaria Report 2014]. In Malaysia, most malaria cases are caused by *Plasmodium falciparum*. An estimated 437 000 of deaths occurred in children aged under 5 years in the WHO African Region. [(WHO), World Malaria Report 2014].

Malaria is a parasitic disease caused by *Plasmodium* species transmitted from the blood of an infected person and passed to a healthy human by a female *Anopheles* mosquito. There are five species of parasites from the genus *Plasmodium* that are pathogenic to humans: *P. falciparum, P. vivax, P. ovale, P. malariae, P. knowlesi*. *Plasmodium*

falciparum is a major parasitic infections disease in the world and continues to cause morbidity and mortality on a large scale in tropical countries and undermining development in the poorest countries of the world. So, most studies have evaluated the activity of compounds on this parasite (Wiesner *et al*., 2003; Greenwood & Mutabingwa, 2002; Wyler, 1993).

So far the battle against this deadly disease has not been successful, because of the appearance of drug-resistant strains. Such a situation has heralded the need for alternative antiplasmodial therapy. The first, the oldest and the most important antimalarial drugs derived from plants had been proven was quinine from *Cinchona* species (Beckmann, 1958) and that is still used today. Hence several research groups are now working to develop new active compounds, a plant-based antimalarial drug isolated from the Chinese plant *Artemisia annua* (Klayman, 1985). Therefore, plants may well prove to be the source of new antimalarial drugs in view of the success with the two important chemotherapeutic agents, quinine and artemisinin, both of which are derived from plants.

In a continuation of our ongoing investigation of traditionally used antimalarial plants of Malay Peninsular, Malaysia, we have evaluated the antiplasmodial activity of *Phoebe tavoyana* (Meissn.) Hk.F (Lauraceae) in our present studies.

CHAPTER 3

METHODOLOGY

3.1 General Experimental Methods

3.1.1 Plant Materials and Extraction

The dried samples of three different species from the Lauraceae family were collected and identified by the botanist and phytochemical survey of Malayan Herbarium, University of Malaya, Malaysia. A summary of the samples are shown in the Table 3.1.

Table 3.1: List of two *Phoebe* and one *Actinodaphne* species with their voucher specimen number.

Species	Local name	Part	Voucher specimen no.	Locality and Date Collected
Phoebe grandis	*Medang Ketanah* or *Tanah*	Bark	KL 4994	Reserved Forest Kuala Tahan, Pahang, Malaysia. 17th May 2001.
Phoebe grandis	*Medang Ketanah* or *Tanah*	Leaves	KL 5440	Reserved Forest Bukit Serting, Negeri Sembilan, Malaysia. 19th Feb 2008.
Phoebe tavoyana	*Medang Rungkoi*	Bark	KL 5225	Reserved Forest Chebar Besar, Kedah, Malaysia. 25th Apr 2006.
Phoebe tavoyana	*Medang Rungkoi*	Leaves	KL 5225	Reserved Forest Chebar Besar, Kedah, Malaysia. 25th Apr 2006.
Actinodaphne sesquipedalis	*Medang payung*	Bark	HIR 011	Reserved Forest Cameron Highland, Pahang, Malaysia. 20th Oct 2011
Actinodaphne sesquipedalis	*Medang payung*	Leaves	HIR 011	Reserved Forest Cameron Highland, Pahang, Malaysia. 20th Oct 2011
Actinodaphne sesquipedalis	*Medang payung*	Fruits	HIR 011	Reserved Forest Cameron Highland, Pahang, Malaysia. 20th Oct 2011.

The ground dried material was extracted at room temperature three times in solvents of increasing polarity beginning with hexane, followed by dichloromethane (CH_2Cl_2) and lastly methanol (MeOH) or extracted with dichloromethane (CH_2Cl_2) solvent by using Soxhlet extractor. The extracts obtained were then concentrated using rotary evaporator under reduced pressure to give dark or brownish viscous crude hexane, CH_2Cl_2 and MeOH extracts. The crude extracts were used for the isolation and biological work.

3.1.2 Solvents

The solvents used in this work were hexane, dichloromethane, ethyl acetate, chloroform and methanol. All solvents are from AR grade except those that are used for bulk extraction (distilled). Other chemicals were hydrochloric acid, ammonium solution and sodium sulphate anhydrous.

3.2 Instruments

3.2.1 Optical Rotation

Optical rotations were determined on a JASCO P-1020 polarimeter with methanol or chloroform as solvent.

3.2.2 Ultraviolet (UV)

UV spectra were obtained using Shimadzu UV-160 PC Ultraviolet-Visible Spectrometer. Solvent used was methanol (CH_3OH) while the wavelength in which the spectrum was recorded is 200 - 400 nm.

3.2.3 Infrared (IR)

Infrared spectra were measured by using Perkin Elmer Spectrum 2000-FTIR Spectrometer. IR spectra were obtained using NaCl cell with solvent chloroform ($CHCl_3$). The adsorption bands were measured in cm^{-1}.

3.2.4 Mass Spectra (MS)

Mass spectra were recorded by using Hewlett Packard HP 6890 Series Mass Selective Detector. Solvent used was chloroform ($CHCl_3$). Mass spectra were also recorded on a Jeol JMS 700 spectrometer using NBA as the matrix for FAB analysis. The Automass Multi Thermofinnigan was used for HR ESI analysis and EIMS spectra were obtained on Shimadzu GC-MS-QP2000A Mass Spectrometer 70 eV. Sometimes mass spectra were obtained using a Shimadzu LCMS-IT-TOF or LCMS-Q-TOF instrument. The solvent used was methanol (MeOH).

3.2.5 Nuclear Magnetic Resonance (NMR Spectra)

Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectra were recorded on either a JEOL FTNMR (400 and 500MHz) or Bruker AVN (400 and 600MHz) spectrometer with tetramethylsilane (TMS) as an internal standard. The solvent used was deuterated chloroform ($CDCl_3$), unless otherwise specified. Chemical shift are quoted in ppm on the δ scale and the signal were described in terms of chemical shift, multiplicity, coupling constant (*J*) are given in Hz where applicable and number of protons. The abbreviations *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *m* (multiplet), *dd* (doublet of doublets), *ddd* (doublet of doublet of doublet), *dddd* (doublet of doublet of doublet of

doublet), *br s* (broad singlet), *qt* (quartet of triplet) and *dq* (doublet of quartet) have been used to express multiplicities.

^{13}C Nuclear Magnetic Resonance (^{13}C-NMR) spectra with off resonance decoupling and DEPT experiments were determined with either a JEOL FTNMR or a Bruker AVN spectrometer operating at 100, 125 and 150 MHz, respectively. The solvent used was deuterated chloroform ($CDCl_3$), unless otherwise specified. The ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC NMR spectra were obtained with the usual pulse sequences.

3.3 Chromatographic Method

Separation and isolation of compounds from the plant crude extract were performed using various chromatographic techniques such as column chromatography (CC), preparative thin layer chromatography (PTLC) and analytical thin layer chromatography (TLC).

3.3.1 Column Chromatography (CC)

The technique employed in this work was gravity column chromatography (CC). The gravity column chromatography was packed with silica gel Silica Gel 60F, 70-230 mesh ASTM (Merck 7734); Silica Gel 60F, 230-400 Mesh ASTM (Merck 9385). The silica was made slurry and poured into a particular column to certain height and the top of the silica was covered with sand. The size and length of the column used depend on the amount of the sample. For the gravity column chromatography, the ratio of silica gel to the sample was approximately 30: 1 for the crude samples. The samples were dry packed with silica gel or dissolved in a minimum amount of solvent and placed on the

top of the sand in the column. The column was then eluted with suitable solvent mixtures, starting with solvent of low polarity. The solvent mixtures used were based on the solvent system of the TLC. Fractions were collected in appropriate bottles or conical flasks.

Another technique used was gel filteration column chromatography which was conducted on Sephadex LH-20. This Sephadex LH-20 employed the same packing technique as the general gravity column chromatography, however, the top of column was not covered with sand and the sample used should be dissolved in appropriate solvent. The column was eluted with solvent from high to medium polarity. The gel Sephadex LH-20 is reusable provided that the gel is properly washed and kept in distilled water or methanol.

3.3.2 Preparative Thin Layer Chromatography (PTLC)

Preparative Thin Layer Chromatography (PTLC) utilised layer of silica gel PF_{254} Art. No. 1.07749.1000 of 0.75 mm thickness, spread on 20 x 20 cm glass plates. The silica gel was applied as slurry (1 part of silica to 2 part of distilled water) dried overnight at room temperature and then activated at 120°C for about 6 hours. The samples were introduced on the plates as continuous streak about 1.5 cm above the base of the plates using capillary tubes. The plates were then developed in the chromatographic jar saturated with appropriate solvent system at room temperature. The bands obtained were visualized under short and long waves of ultraviolet light, and the relevant bands were scraped off.

3.3.3 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was performed on commercially available Merck aluminium supported silica gel $60F_{254}$ TLC sheets (Merck 1.05554.0001); Glass supported silica gel $60F_{254}$ TLC plates (Merck 1.05715.0001).

Fractions were monitored by TLC and appropriate fractions were combined and when necessary subjected to further separation by rechromatography or preparative TLC. TLC was routinely used to detect and separate the various compounds or alkaloids. The crude extracts, fractions from chromatography and isolated pure alkaloids were examined by TLC. The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated chromatographic tanks with various solvent systems at room tempetature. The alkaloidal spots were visualized by examination of the TLC plates under UV light (254 and 365 nm), followed by spraying with Dragendrorff's reagent that formed orange spots.

3.4 Reagents

Mayer's and Dragendorff's Reagent were used for alkaloid screening to identify the presence of the alkaloids and alkaloids detection (TLC).

3.4.1 Mayer's Reagent (Potassium mercuric iodide)

Mayer's reagent **:** A solution of mercury (II) chloride (1.4 g) in distilled water (60 ml) was poured into a solution of potassium iodide (5.0g) in distilled water (10ml). The mixture was then made up to 150 ml solution.

Mayer's test **:** A positive test is indicated by formation of a white precipitate when the aqueous layer (acidified) is treated with 2-3 drops of Mayer's reagent.

3.4.2 Dragendorff's Reagent (Potassium bismuth iodide)

Solution A **:** 0.85 g of bismuth (III) nitrate was mixed with glacial acetic acid (10 ml) and distilled water (40 ml).

Solution B **:** Potassium iodide (8.0g) was dissolved in distilled water (20 ml).

Solution A and solution B were mixed to give the stock solution. The stock solution must be an equal volume of both solutions. The spray reagent was prepared by diluting 20 ml of stock solution with acetic acid (20 ml) and distilled water (60 ml).

Dragendorff's test **:** A positive result is indicated by the formation of orange spots.

3.4.3 Detector Reagent – Vanilin Sulphuric Acid Vapour

About 1.0 g vanilin in 10 mL of concentrated H_2SO_4 was added upon cooling to 90 mL of ethanol before spraying onto the TLC plate. The TLC plate was then heated at 50°C untill full development of colours had been observed. The occurence of blue, purple, dark green, grey or brown indicated the presence of terpenes, steroids and limonoids.

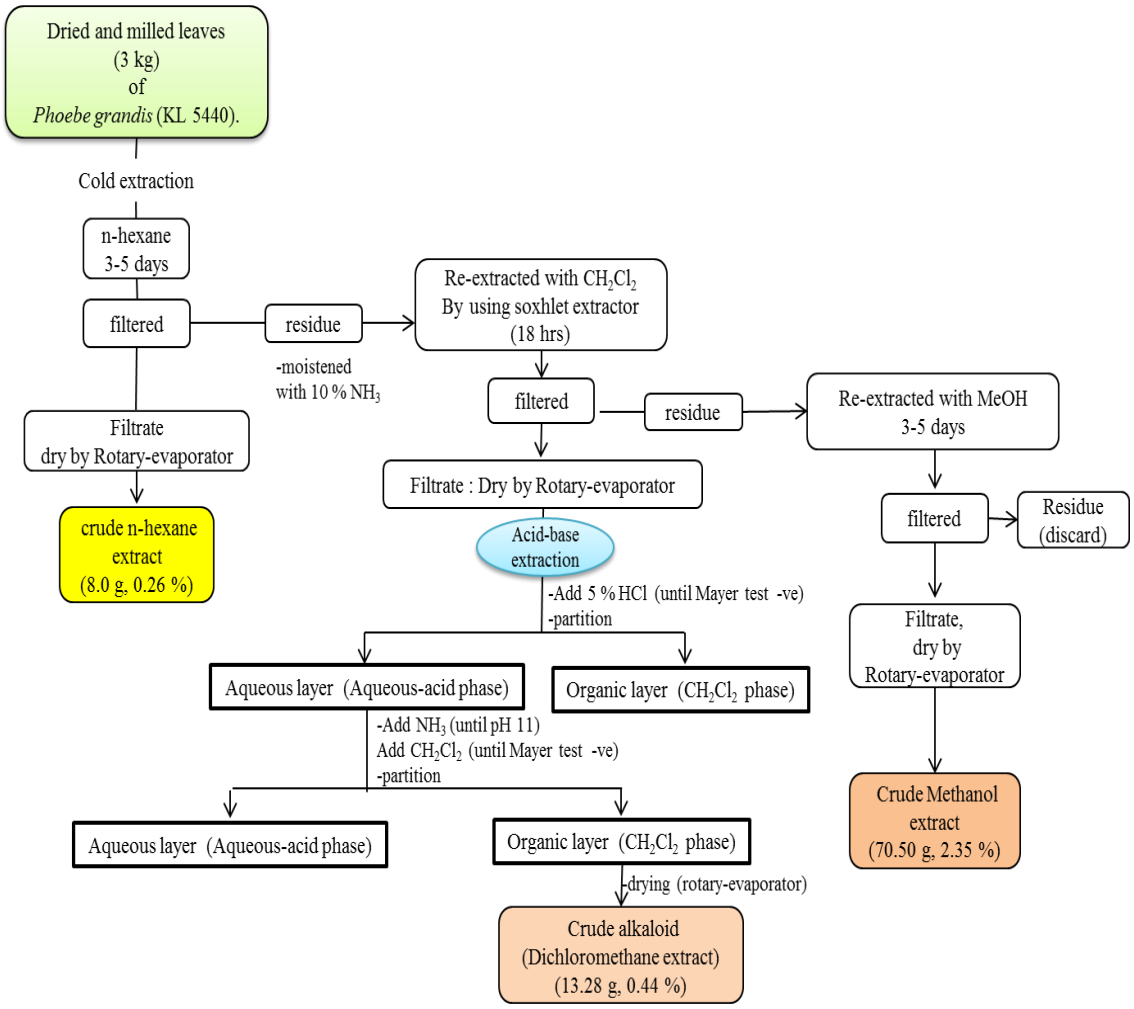
3.5 Preparation of crude extracts from three different plant species for preliminary biological screening.

Each sample from three species of ***Phoebe grandis, Phoebe tavoyana*** and ***Actinodaphne sesquipedalis*** plants (20 g) were ground individually into fine powder and extracted sequentially at room temperature with similar procedure as described earlier (page 51) in solvents of different polarity beginning with hexane, followed by dichloroform (CH_2Cl_2) and lastly methanol (MeOH). The extract were filtered and evaporated under reduced pressure to give plant extracts. Each crude extract (100 mg/mL) was screened for selected bioassays such as cytotoxicity, antioxidant, antibacterial and antiplasmodial activities.

3.6 Extraction and Isolation of Chemical constituents

3.6.1 Chemical constituents from leaves of *Phoebe grandis* (Nees) Meer - KL 5440

The dried ground leaves 3 kg of ***Phoebe grandis*** was extracted successively with hexane, CH_2Cl_2 (acid-base process) and MeOH to give 8.0 g (0.26 %), 13.28 g (0.44 %) and 70.5 g (2.35 %) of dark viscous extracts, respectively. Refer Scheme 3.1.



Scheme 3.1: Extraction and isolation of chemical constituents from the leaves of *Phoebe grandis* (KL 5440).

3.6.1.1 Extraction and fractionation of dichloromethane extract (crude alkaloid)

Dried and milled leaves of ***Phoebe grandis*** **(Nees) Merr.** (3.0 kg) were first defatted with hexane (7 L) for 3 days at room temperature, than the extracts were filtered. The residue were moistened with 10 % of NH_4OH, and exhaustively extracted with CH_2CI_2 using a Soxhlet extractor for about 18 h. The CH_2CI_2 extract was reduced to 500 mL followed by acidic extraction using 5% HCI until the Mayer's test was negative. The combined extracts were then made alkaline with concentrated ammonia solution to pH10–11 and re-extracted with CH_2CI_2. The CH_2Cl_2 fractions were washed with distilled H_2O and dried over anhydrous sodium sulphate. The dichloromethane extract

was evaporated to dryness under reduced pressure to give the crude extract. The plant residue was extracted with methanol and the methanol was evaporated to dryness and then acidified using 5 % hydrochloric acid solution and left to stand overnight. The acid solution was then filtered and made alkaline with 10% ammonia solution and reextracted with dichloromethane. The dark residue obtained after washing, drying and evaporating to dryness was added to the crude alkaloid obtained from the dichloromethane extracts to yield (13.28 g, 0.44 %) of crude alkaloids.

The dichloromethane crude extract (8.0 g, 0.27 %) was subjected to column chromatography over silica gel using various ratios of CH_2CI_2 and MeOH (100: 0, 98: 2, 97: 3, 96: 4, 95: 5, 94: 6, 93: 7, 92: 8, 91: 9, 90: 10, 88: 12, 80: 20, 70: 30, 60: 40, 50: 50) and finally with pure 100 % MeOH. The collected fractions were grouped into a series of fractions, monitored with TLC. Each series were then treated separately to isolate and purify its alkaloid by extensive column chromatography followed by preparative TLC.

3.6.2 Chemical constituents from bark of *Phoebe grandis* (Nees) Meer - KL 4994

The air dried stem barks of *Phoebe grandis* (Nees) Meer (1 kg) was extracted successively with hexane, repeated with dichloromethane (CH_2Cl_2) and were then subjected to the acid-base process and methanol (MeOH), respectively. The weights of hexane, CH_2Cl_2 and MeOH crude extract obtained were 2.32 g (0.23 %), 5.02 g (0.50 %) and 15.46 g (1.55 %), respectively. (Extraction and isolation of of chemical constituents was described earlier. Refer Scheme 3.1 of *Phoebe grandis* (KL 5440)).

3.6.2.1 Extraction and fractionation of hexane extract

The air dried stem barks of ***Phoebe grandis*** **(Nees) Meer** (1000g) were ground into powder. It was soaked at room temperature in hexane for three days and repeated three times. After filtering the extract through Whatman No.1 filter paper, the solvent was concentrated using rotary evaporator at room temperature.

The hexane extract (1.32 g, 0.13 %) was coated with silica gel and fractionated by gravity column chromatography (CC 1) eluting with 100 % of hexane and followed by mixtures of solvents, hexane : ethyl acetate and ethyl acetate : methanol (gradient polarity). The eluents of 100 mL was collected in a conical flask which was then concentrated. The fractions collected were combined depending on the similar spot detected under UV light.

3.6.2.2 Extraction and fractionation of dichloromethane extract (crude alkaloid)

About 1 kg of dried and milled stem barks of the plant were first defatted in hexane for 3 days at room temperature then filtered and air-dried for 24 hours, and the solvent evaporated to dryness. After being dried, the barks samples were sprinkled with 15% ammonia (NH_3) solution and left to soak overnight. They were then exhaustively extracted with dichloromethane (CH_2Cl_2) solvent by Soxhlet extractor for about 18 hours. The CH_2Cl_2 extract were concentrated to about 500 ml by using the rotary evaporator. Then, re-extracted with 5% hydrochloric acid (HCl) until Mayer's test is negative. The combined extracts were then basified with concentrated ammonia solution to pH 10-11 and re-extracted with CH_2CI_2. The CH_2Cl_2 extracts were washed with distilled H_2O and followed by sodium chloride solution and finally dried over

anhydrous sodium sulphate. The solvents were evaporated to dryness to yield 5.02 g of crude alkaloid (0.50 % wt/wt). The crude alkaloid fraction was obtained as a dark gummy residue.

The dichloromethane crude extract or crude alkaloids (4.0 g, 0.4 %) was fractionated on a column of silica gel 60 (70 – 230 Mesh, Merck). Elution was done using CH_2CI_2 gradually enriched with MeOH (100: 0, 98: 2, 97: 3, 96: 4, 95: 5, 94: 6, 93: 7, 92: 8, 91: 9, 90: 10, 88: 12, 80: 20, 70: 30, 60: 40, 50: 50) and finally with pure 100 % MeOH. The collected fractions were grouped into a series of fractions, monitored with TLC. Each series were then treated separately to isolate and purify its alkaloid by extensive column chromatography followed by preparative TLC.

3.6.3 Chemical constituents from leaves of *Phoebe tavoyana* (Meissn.) Hk.F. (KL 5225)

The dried ground leaves 2700 g of ***Phoebe tavoyana*** was extracted exhaustively with hexane followed by CH_2Cl_2 using Soxhlet extractor for 17 hrs. and finally the residue was extracted with MeOH to give 5.0 g (0.19 %), 10.5 g (0.39 %) and 50.5 g (1.87 %) of dark viscous extracts, respectively. (Extraction and isolation of of chemical constituents was described earlier. Refer Scheme 3.1 of *Phoebe grandis* (KL 5440)).

3.6.3.1 Extraction and fractionation of dichloromethane extract (crude alkaloid)

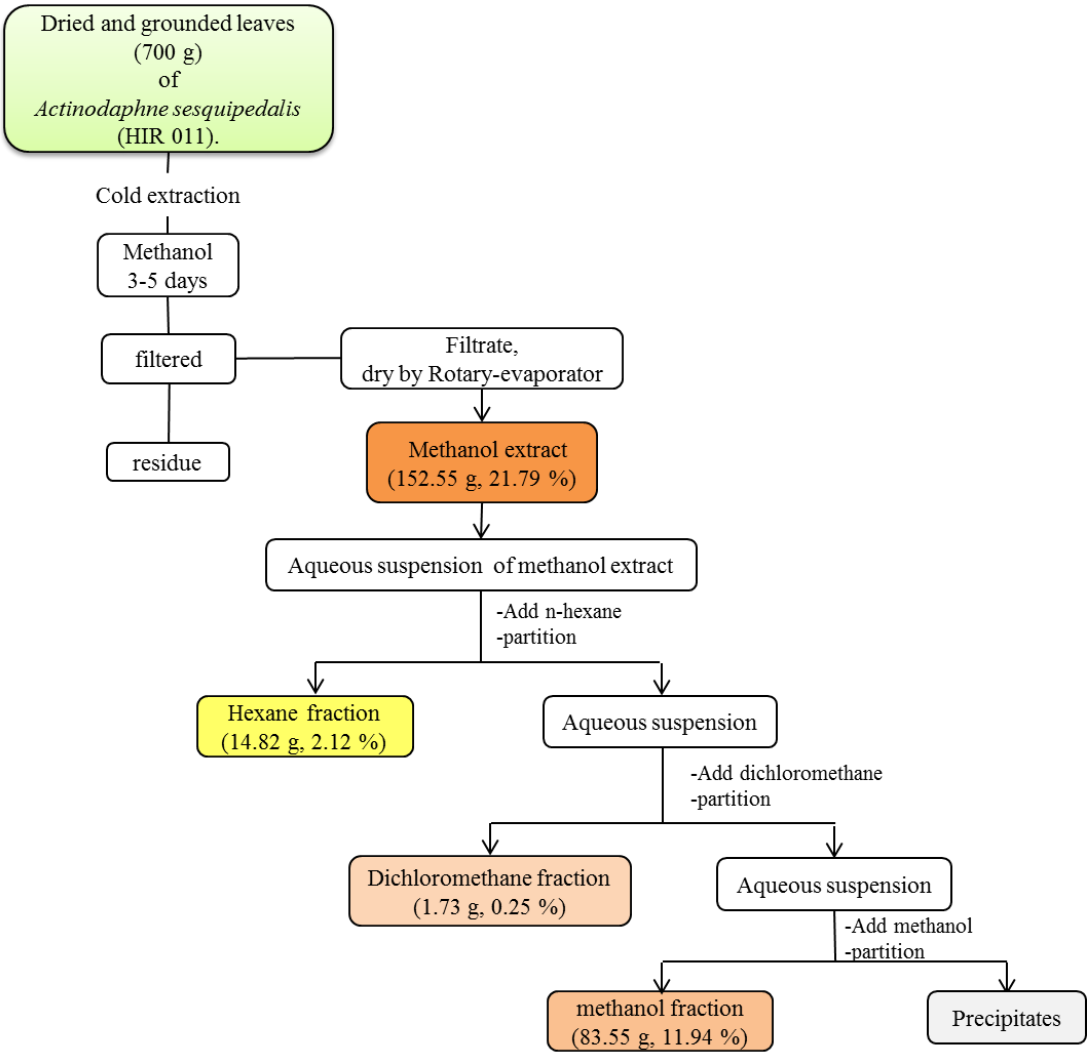
Extraction of alkaloids was carried out in the usual manner, which has been described earlier in detail in section [3.6.2.2] for bark of *Phoebe grandis* (Nees) Merr. and gave 10.5 g (0.39 %) of crude alkaloid. Several alkaloids were isolated from the

dichloromethane extract, while separation of the hexane and methanol extracts did not give any compounds.

The dichloromethane extract or crude alkaloid (8.0 g, 0.3%) was coated with silica gel and fractionated by gravity column chromatography (CC) eluting with 100 % of dichloromethane and followed by mixtures of solvents, dichloromethane: methanol (gradually enriched with MeOH). The eluents of 50 mL was collected in a conical flask which was then concentrated. The fractions collected were combined depending on the similarity of spots on TLC detected under UV light. Each series were then treated separately to isolate and purify the compound by extensive column chromatography and TLC.

3.6.4 Chemical constituents from leaves of *Actinodaphne sesquipedalis* (HIR 011)

Dried and ground leaves (700 g) of ***Actinodaphne sesquipedalis*** were successively extracted with MeOH at room temperature. The combined MeOH extracts were evaporated under reduced pressure to give a dark residue (152.55 g), which was partitioned between n-hexane (14.82 g; 2.12 %), dichloromethane (1.73 g; 0.25 %) and MeOH (83.55 g; 11.94 %). TLC investigations indicated a high concentration of alkaloids in the MeOH extract. Several alkaloids and steroids were isolated from the hexane and methanol extracts; while, no separation was conducted on dichloromethane extract due to limited amount of dichloromethane extract obtained. Refer Scheme 3.2.



Scheme 3.2: Extract and partitioning of the crude leaves extract of *Actinodaphne sesquipedalis* (HIR 011).

3.6.4.1 Extract preparation

About 700 grams of grounded leaves of ***Actinodaphne sesquipedalis*** macerated using methanol solvent. Maceration performed in Erlenmeyer flask with a magnetic stirrer speed of 700 rpm for 1 hour. This process was performed many times until the methanol layer is colourless. The extract then was evaporated to dryness under reduced pressure (below 40˚C) to yield crude methanolic extract.

3.6.4.2 Partitioning of the crude extracts

The crude methanolic extract solution is partitioned using hexane 50 mL. Partitioning was done many times until a colourless hexane layer appeared. Two layers were formed, hexane extracts (non-polar fraction) and methanol extracts. Furthermore, the methanol extract obtained was partitioned again using 50 mL dichloromethane. Partitioning was done many times and stopped until the dichloromethane layer is colourless. By this process, dichloromethane extract (semipolar fraction) and methanol extract (polar fraction) were obtained. All the collected filtrates were evaporated using rotary evaporator.

(a) Fractionation of hexane extract

The hexane extract (9.0 g, 1.29 %) was fractionated by column chromatography using hexane with a gradient on ethyl acetate up to 100 %, followed by methanol, to give 20 fractions of 125 mL in a conical flask. The fractions collected were combined on similar spot as monitored and evaluated by TLC.

(b) Fractionation of methanol extract

The methanol crude extract (10.0 g, 1.43 %) was fractionated by column chromatography (Sephadex LH-20 used as stationary phase) and the mobile phase used was methanol to give 50 fractions. The collected fractions with the same profile spots were grouped into a series of fractions, monitored by TLC. Each series were then treated separately to isolate and purify its chemical constituents by extensive column chromatography followed by preparative TLC.

3.6.5 Chemical constituents from fruits of *Actinodaphne sesquipedalis* - HIR 011

The air-dried ground, fruits (400 g) of ***Actinodaphne sesquipedalis*** were initially extracted with MeOH (1 L) at room temperature, three days repeated three times. The solvent was evaporated under reduced pressure to give a dark residue (124.57 g), which was partitioned between n-hexane (9.89 g), dichloromethane (1.93 g) and MeOH (100.00 g). (Extraction and partitioning of crudes was described earlier. Refer Scheme 3.2 of *Actinodaphne sesquipedalis* (HIR 011).

3.6.5.1 Extract preparation

The air-dried ground, ***Actinodaphne sesquipedalis*** fruits (400 g) was macerated by soaking in 80 % methanol (1 L, 72 h) at room temperature. It was repeated thrice and the resultant extracts were combined and then concentrated *in vacuo* using rotary evaporator at 35°C to give crude methanolic extract. The extract was air-dried and weighed.

3.6.5.2 Partitioning of the crude extracts

The crude methanolic extract (124.57 g) was dissolved in 500 mL of methanol. The resultant solution was partitioned with 250 mL thrice each in order of their increasing polarities (n-hexane and dichloromethane). All of three fractions obtained were filtered one after the other using Whatman No.1 filter paper sheets. Each extract was then concentrated over a water bath at 35°C by the evaporation of various solvents from the extracts after which phytochemical screening was conducted on each fraction and the

methanolic extract which was having most of the metabolite was taken for further analysis.

(a) Fractionation of hexane extract

The hexane extract (7.0 g, 1.75 %) was fractionated by column chromatography used a silica gel 60 as the stationary phase and for the mobile phase used a mixture of hexane - ethyl acetate with increasing polarity (gradient elution) up to 100 %, followed by methanol, to give 15 fractions of 100 mL in a conical flask. The fractions collected were combined on similar spot as judged by TLC.

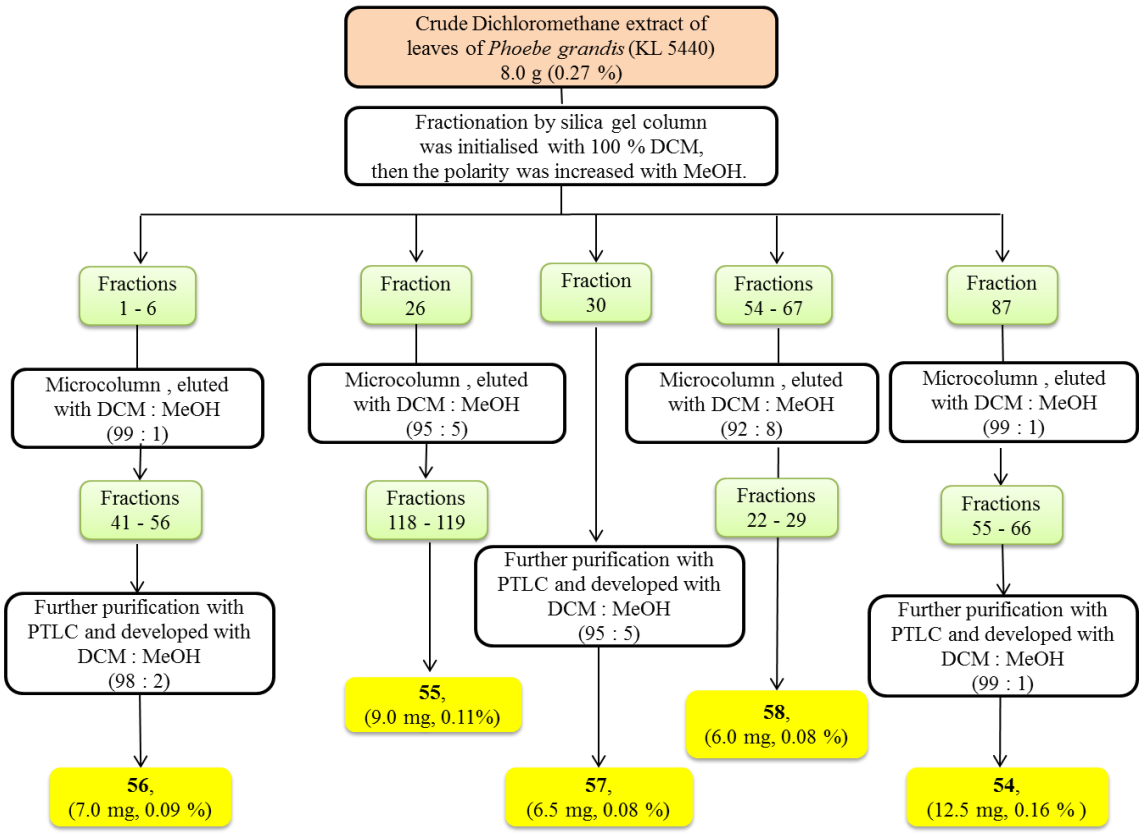
(b) Fractionation of methanol extract

The methanol crude extract (10.0 g, 2.5 %) was fractionated by column chromatography (Sephadex LH-20) and eluted with methanol to give 25 fractions of 200 mL each. The collected fractions were grouped into a series of fractions, monitored with TLC. Each series were then treated separately to isolate and purify its chemical constituents by extensive column chromatography followed by preparative TLC.

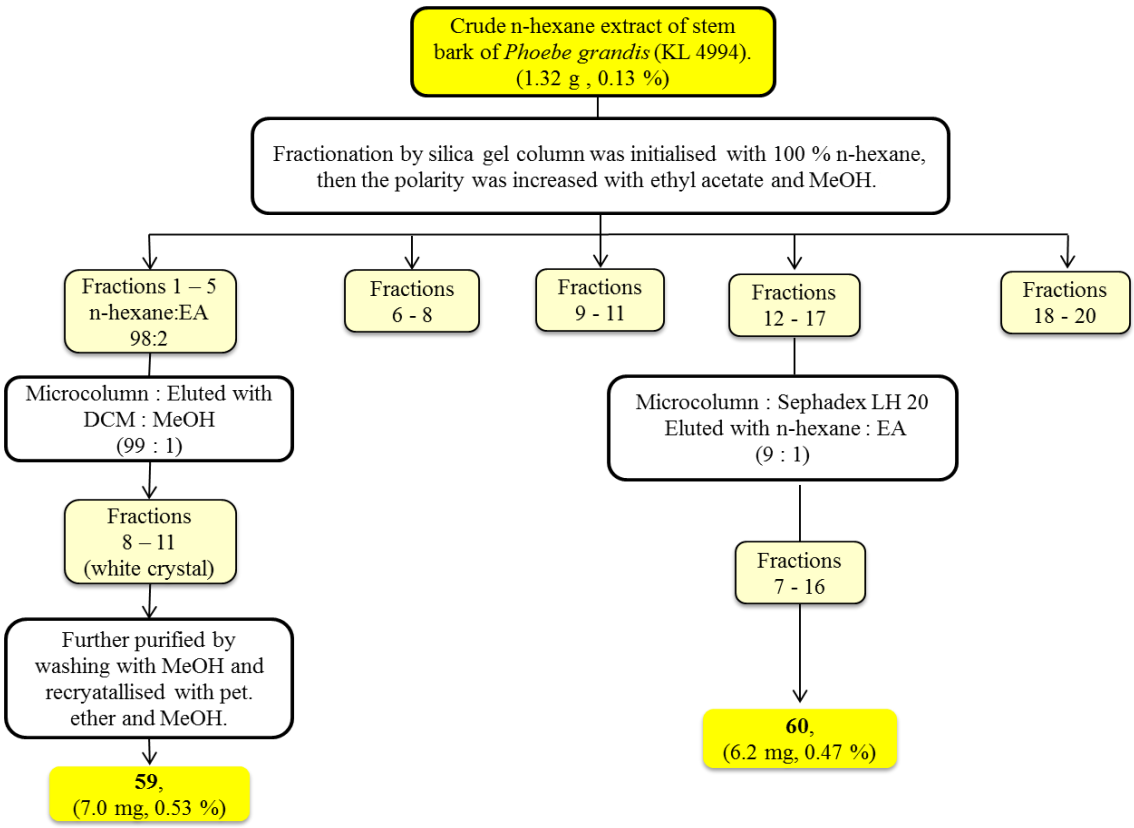
3.7 Isolation and purification

The crude samples were isolated using column chromatography with silica gel 60 or Sephadex LH-20 as stationary phase. The solvent systems used for chromatography were n-hexane or dichloromethane with increasing portion of methanol (gradient elution system). The example of the ratio of solvent system between CH_2Cl_2 and CH_3OH were

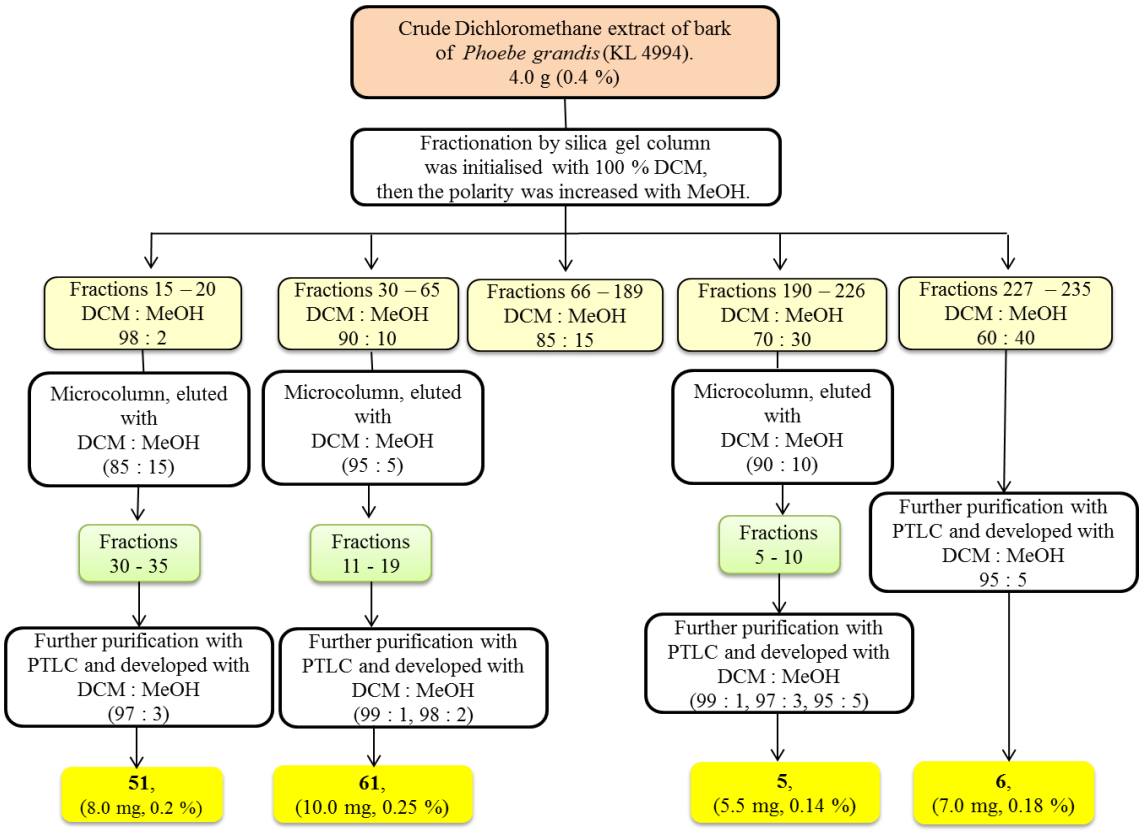
(100 : 0; 99 : 1; 98 : 2; 97 : 3; 96 : 4; 95 : 5; 93 : 7; 90 : 10; 85 : 15; 80 : 10; and 50 : 50). Fractions were collected and each fraction was tested with aluminium TLC plate for their alkaloids and non-alkaloids. The alkaloid spot were first detected by UV light (254 and 366 nm) and confirmed by spraying with Dragendroff's reagent. Fraction having spots with the same R_f values were combined and treated as a group. The combined groups were isolated again with CC or preparative TLC to purify the compounds. The isolation and purification process are given in the Scheme 3.3 – 3.10.



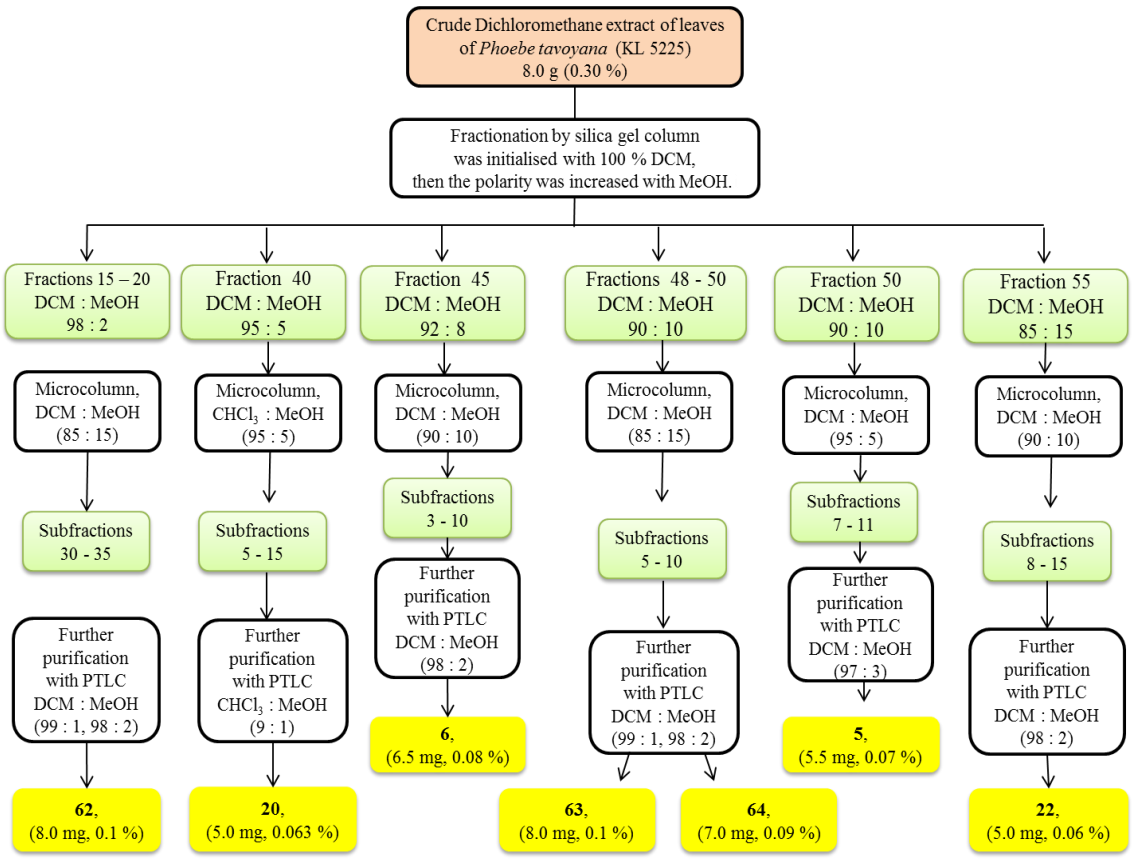
Scheme 3.3: Isolation of alkaloids from crude dichloromethane leaves extract of *Phoebe grandis* (KL 5440).



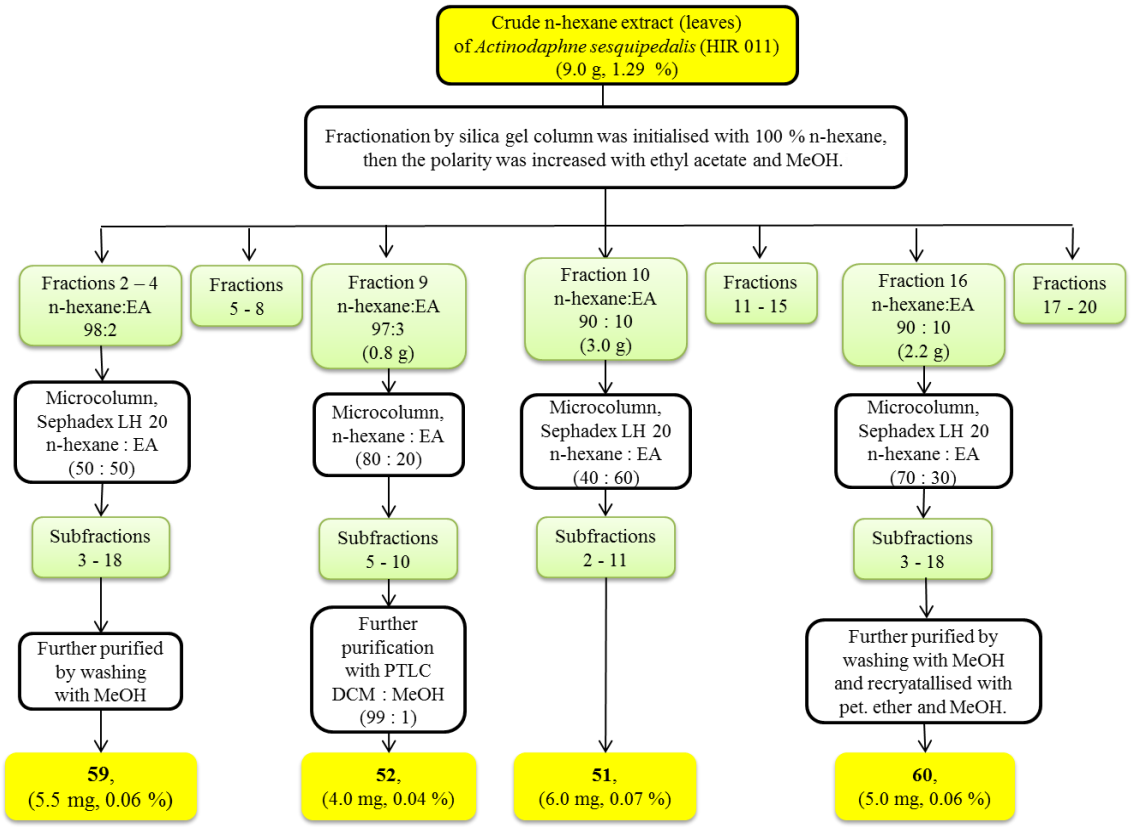
Scheme 3.4: Isolation of alkaloids from crude n-hexane bark extract of *Phoebe grandis* (KL 4994).



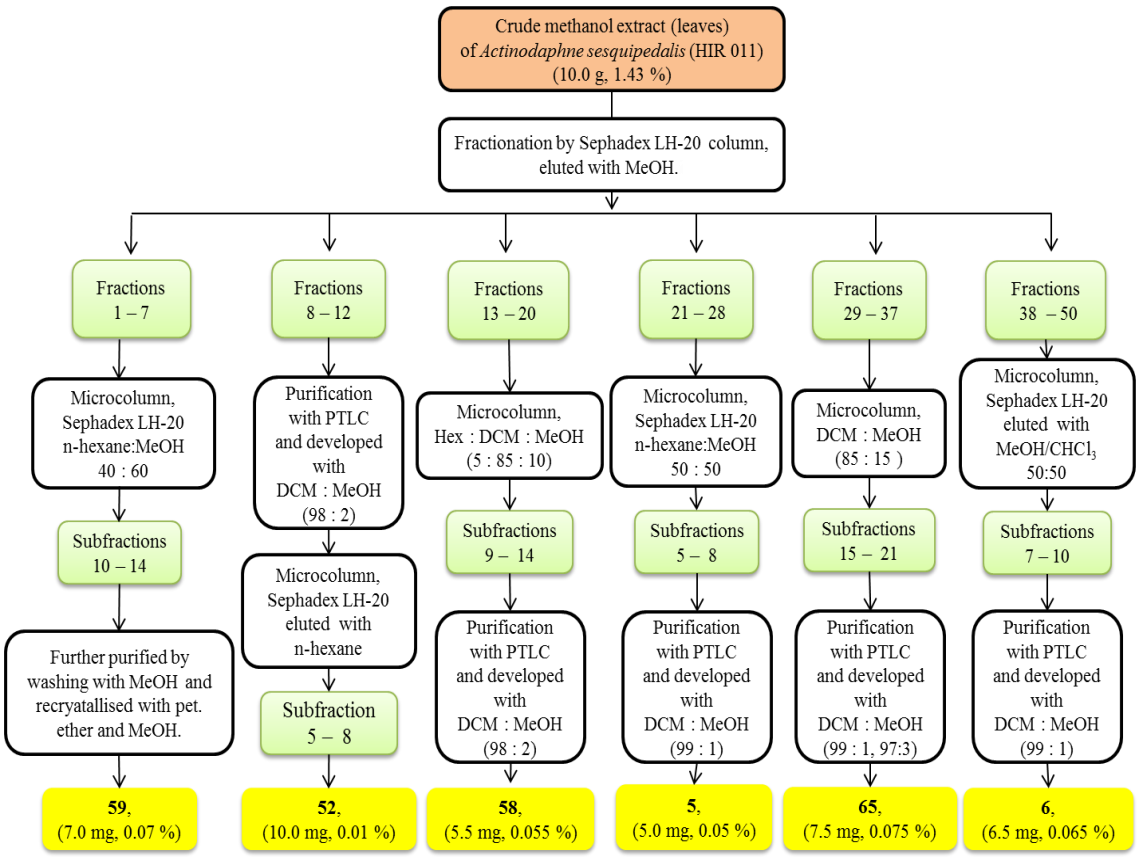
Scheme 3.5: Isolation of alkaloids from crude dichloromethane bark extract of *Phoebe grandis* (KL 4994).



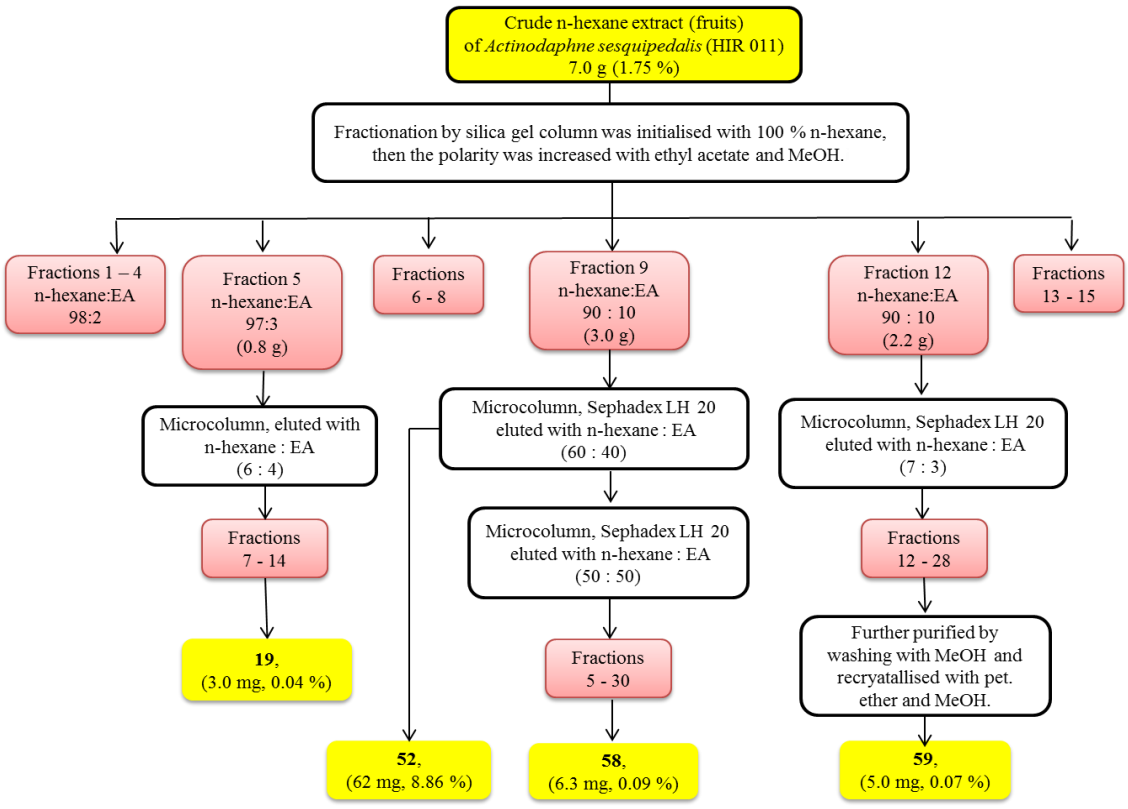
Scheme 3.6: Isolation of alkaloids from crude dichloromethane leaves extract of *Phoebe tavoyana* (KL 5225).



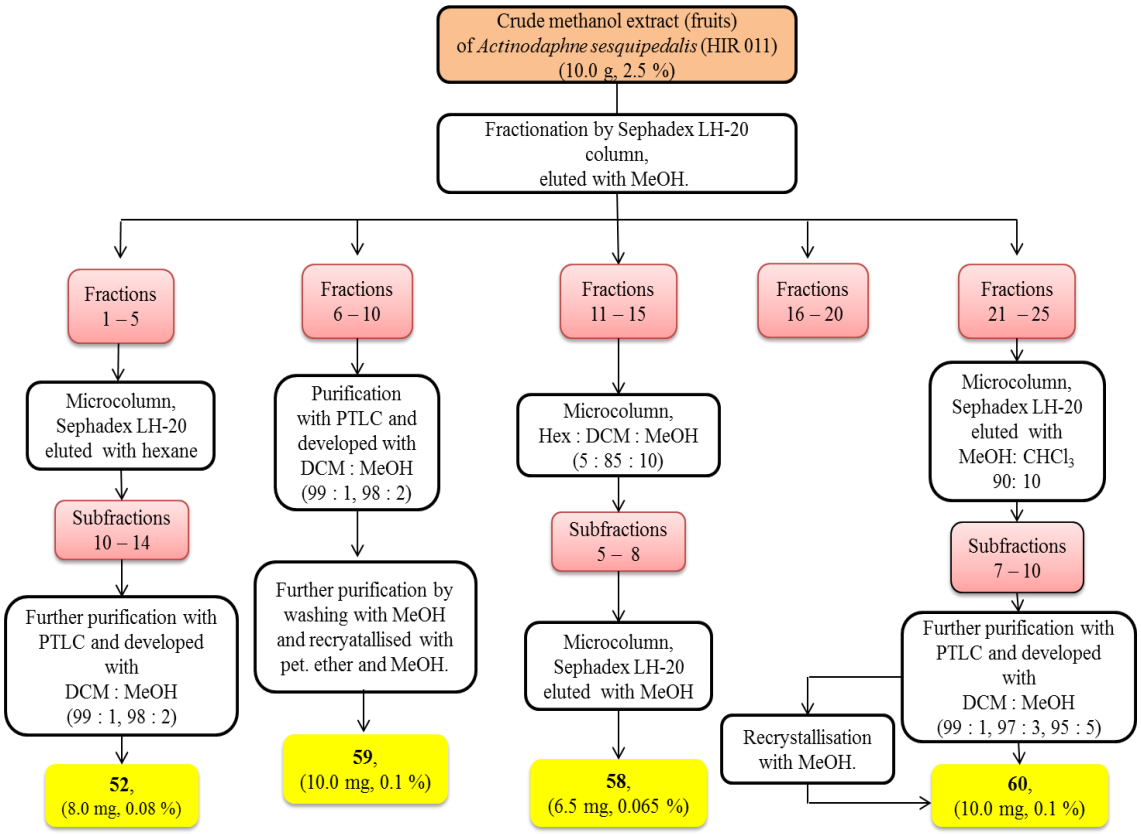
Scheme 3.7: Isolation and purification of chemical constituents from crude n-hexane leaves extract of *Actinodaphne sesquipedalis* (HIR 011).



Scheme 3.8: Isolation of chemical constituents from crude methanol leaves extract of *Actinodaphne sesquipedalis* (HIR 011).



Scheme 3.9: Isolation and purification of chemical constituents from crude n-hexane fruits extract of *Actinodaphne sesquipedalis* (HIR 011).



Scheme 3.10: Isolation and purification of chemical constituents from crude methanol fruits extract of *Actinodaphne sesquipedalis* (HIR 011).

3.7.1 Physical and Spectral Data of Isolated Constituents

3.7.1.1 *Phoebe grandis* (leaves) Alkaloids – KL 5440

Lysicamine (54)

Fraction 87, with the eluent of CH_2Cl_2 : CH_3OH through column chromatography was obtained from fractionation of dichloromethane extract of leaves of *Phoebe grandis* (Nees) Meer. Further purification by re-column of fraction 87 in solvent system 99 : 1 ratio of CH_2Cl_2 : MeOH gave 40.5 mg of yellow amorphous solid. Fractions of 55 - 66 from microcolumn gave the compound that showed one yellow spot with R_f = 0.7 on TLC after developed in solvent system 98 : 2 ratio of CH_2Cl_2 : MeOH. The compound was identified as lysicamine (**54**), (12.5 mg, 0.16 %) a known compound by comparison of their spectra data with literature values. (Refer Scheme 3.3)

Physical data:

$C_{18}H_{13}NO_3$, yellow amorphous solid. $[\alpha]^{29}_D$ = - 25° (c= 0.5, $CHCl_3$). Yield: 0.16 %

UV MeOH λ_{max} nm (log ε): 236, 267, 360 and 396.

IR ν_{max} cm^{-1} ($CHCl_3$): 1659 cm^{-1} conjugated carbonyl absorption.

LCMS Q- TOF *m/z*: 292.0963 $[M+H]^+$. (calcd for $C_{18}H_{14}NO_3$, 292.0929).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 7.21 (1H, *s*, H-3), 7.78 (1H, *d*, *J* = 5.2, H-4), 8.88 (1H, *d*, *J* = 5.2, H-5), 8.57 (*dd*, *J* = 8.4, 1.4, H-8), 7.56 (1H, *dt*, *J* = 8.5, 1.6, H-9), 7.75 (1H, *dt*, *J* = 8.5, 1.6, H-10), 9.16 (1H, *dd*, *J* = 8.4, 1.4, H-11), 4.00 (3H, *s*, 1-OMe), 4.08 (3H, *s*, 2-OMe).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 151.0 (C-1), 118.8 (C-1a), 121.2 (C-1b), 155.8 (C-2), 105.4 (C-3), 134.5 (C-3a), 122.6 (C-4), 144.0 (C-5), 144.3 (C-6a), 181.7 (C-7), 133.3 (C-7a), 127.8 (C-8), 127.9 (C-9), 133.4 (C-10), 127.4 (C-11), 131.04 (C-11a), 59.7 (1-OCH_3), 55.2 (2-OCH_3).

Litsericinone (55)

Fraction 26 from the big column was further purified on silica gel microcolumn eluted with increasing amount of MeOH in dichloromethane, yielding 150 fractions of 20 mL each that were evaluated and pooled according to TLC analysis, to give 8 subfractions. Subfractions 118-119 gave a pure compound of **55**, litsericinone (9.0 mg, 0.11 %). It was isolated for the first time as natural compounds. (Refer Scheme 3.3)

Physical data:

$C_{17}H_{19}NO_3$, yellow amorphous; $[\alpha]^{29}_D$ = -25°(c=0.5, $CHCl_3$). Yield: 0.11 %

UV MeOH λ_{max} nm (log ε): 300, 236 and 207nm (indicated the existence of a conjugated system).

IR ν_{max} cm^{-1} ($CHCl_3$): 3399, 2951, 1712, 1248, 934 cm^{-1}.

LCMS-IT-TOF, ESI m/z: 286.1421 $[M+H]^+$ (calcd for $C_{17}H_{20}NO_3$, 286.1432).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.51 (1H, *s*, H-3), 2.87 (1H, *m*, H-4β), 2.75 (1H, *m*, H-4α), 3.55 (1H, *m*, H-5β), 3.16 (1H, *m*, H-5α), 4.26 (1H, *dd*, *J* = 6.9, 9.8 Hz, H-6a), 2.73 (1H, *m*, H-7β), 1.87 (1H, *m*, H-7α), 2.68 (1H, *m*, H-8β), 2.41 (1H, *m*, H-8α), 2.50 (1H, *m*, H-9β), 1.91 (1H, *m*, H-9 α), 2.46 (1H, *m*, H-11β), 2.45 (1H, *m*, H-11α), 2.10 (1H, *m*, H-12β), 2.00 (1H, *m*, H-12α), 5.92 (1H, *d*, *J*=1.2 (O-CH_2-O), 5.88 (1H, *d*, *J*=1.2 (O-CH_2-O).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 141.1 (C-1), 132.4 (C-1a), 123.9 (C-1b), 148.9 (C-2), 106.9 (C-3), 126.7 (C-3a), 25.1 (C-4), 43.9 (C-5), 56.8 (C-6a), 44.1 (C-7), 46.2 (C-7a), 38.5 (C-8), 36.2 (C-9), 211.1 (C-10), 38.9 (C-11), 34.3 (C-12), 100.9 (O-CH_2-O).

8,9,11,12-tetrahydromecambrine (56)

Fractions 1 - 6 from the big column was further purified on silica gel microcolumn eluted with increasing amount of MeOH in dichloromethane, yielding 70 fractions of 30 mL each that were evaluated and pooled according to TLC analysis, resulting in 9 fractions. Subfractions 41 - 56 was further purified on preparative TLC eluted with dichloromethane–methanol (99 : 1, v/v, three times) yielding a pure compound **56**, 8,9,11,12-tetrahydromecambrine (7.0 mg, 0.09 %)**.** The compound also isolated for the first time as natural compounds. (Refer Scheme 3.3)

Physical data:

$C_{18}H_{21}NO_3$, yellow amorphous solid. $[\alpha]^{29}_D$ = -25°(c=0.5, $CHCl_3$). Yield: 0.09 %

UV MeOH λ_{max} nm (log ε): 203.0 nm

IR ν_{max} cm^{-1} ($CHCl_3$): 2930, 1712, 1470, 1254, 1055, 944, 754 cm^{-1}.

LCMS-IT-TOF *m/z*: 300.1596 $[M+H]^+$, (calcd for $C_{18}H_{22}NO_3$, 300.1521).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.49 (1H, *s*, H-3), 2.92 (1H, *m*, H-4ax), 2.72 (1H, *m*, H-4eq), 3.09 (1H, *m*, H-5ax), 2.45 (1H, *m*, H-5eq), 3.30 (1H, br *s*, H-6a), 2.59 (1H, *m*, H-7ax), 1.75 (1H, *m*, H-7eq), 2.14 (1H, *m*, H-8ax), 2.02 (1H, *m*, H-8eq), 2.47 (1H, *m*,

H-9ax,eq), 2.70 (1H, *m*, H-11ax), 2.43 (1H, *m*, H-11eq), 2.50 (1H, *m*, H-12ax), 1.93 (1H, *m*, H-12eq), 5.90 (1H, *d*, *J*=1.24, O-CH_2-O), 5.86 (1H, *d*, *J*=1.24, O-CH_2-O).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 140.7 (C-1), 134.2 (C-1a), 124.5 (C-1b), 148.2 (C-2), 106.5 (C-3), 126.9 (C-3a), 27.4 (C-4), 55.0 (C-5), 65.7 (C-6a), 44.5 (C-7), 46.0 (C-7a), 34.6 (C-8), 39.0 (C-9), 211.7 (C-10), 38.6 (C-11), 36.5 (C-12), 100.6 (O-CH_2-O).

Hexahydromecambrine A (57)

Fraction 30 was subjected to preparative TLC eluted with dichloromethane–methanol (95:5, v/v, three times) affording a pure compound of **57**, hexahydromecambrine A (6.5 mg, 0.08 %). (Refer Scheme 3.3)

Physical data:

$C_{18}H_{23}NO_3$, amorphous solid. $[\alpha]_D^{29}$ = +100° (c=0.5, $CHCl_3$). Yield: 0.08 %.

UV MeOH λ_{max} nm (log ε): 300, 245 and 265nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3391, 1254 and 929 cm^{-1}.

LCMS-Triple-TOF *m/z*: 302.9177 $[M+H]^+$, (calcd for $C_{18}H_{24}NO_3$, 302.9167).

^{1}H NMR δ (600 MHz, $CDCl_3$, *J*/Hz): 6.46 (1H, *s*, H-3), 2.93 (1H, *m*, H-4ax), 2.71 (1H, *m*, H-4eq), 3.11 (1H, *m*, H-5ax), 2.46 (1H, *m*, H-5eq), 3.26 (1H, *m*, H-6a), 2.44 (1H, *m*, H-7ax), 1.58 (1H, *m*, H-7eq), 2.03 (1H, *m*, H-8ax), 1.54 (1H, *m*, H-8eq), 2.41 (1H, *m*, H-9ax), 1.46 (1H, *m*, H-9eq), 4.00 (1H, br, *m*, H-10ax), 1.75 (2H, *m*, H-11), 1.25 (2H, *m*, H-12), 2.39 (3H, *s*, NCH_3), 5.90 (1H, *d*, *J*=1.2, O-CH_2-O), 5.86 (1H, *d*, *J*=1.2, O-CH_2-O).

^{13}C NMR ppm (150 MHz, $CDCl_3$): 148.1 (C-1), 129.0 (C-1a), 131.0 (C-1b), 140.8 (C-2), 105.9 (C-3), 124.0 (C-3a), 27.3 (C-4), 54.9 (C-5), 65.7 (C-6a), 44.3 (C-7), 46.6 (C-7a), 30.2 (C-8), 31.7 (C-9), 67.1 (C-10), 31.0 (C-11), 29.7 (C-12), 43.2 (NCH_3), 100.5 (O-CH_2-O).

Dicentrinone (58)

Fractions 54 - 67 were subjected to successive silica gel microcolumn eluted with increasing amount of MeOH in dichloromethane, (100 : 0 to 30 : 70, v/v), to give fraction 22 – 29 as **58**, dicentrinone (6.0 mg, 0.08 %) with the eluent CH_2Cl_2-CH_3OH; 92 : 8. (Refer Scheme 3.3)

Physical data:

$C_{19}H_{13}NO_5$, yellow fine needles. Yield: 0.08 %

m.p. : 300°C.

UV MeOH λ_{max} nm (log ε): 218, 257, 270, 308, 347 and 399nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 2916 and 2849 cm^{-1} (CH-aromatic), 1725 (conjugated C=O), 1276 and 995 cm^{-1} (methylenedioxy).

LCMS Triple TOF-MS m/z: 336.0863 $[M+H]^+$, (calcd for $C_{19}H_{14}NO_5$, 336.0827).

^{1}H-NMR δ (600 MHz, $CDCl_3$, *J*/Hz): 7.11 (1H, *s*, H-3), 7.72 (1H, *d*, *J*=5.2, H-4), 8.86 (1H, *d*, *J*=5.2, H-5), 7.97 (1H, *s*, H-8), 7.99 (1H, *s*, H-11), 4.00 (3H, *s*, 9-OCH_3), 4.06 (3H, *s*, 10-OCH_3), 6.34 (2H, *s*, O-CH_2-O).

^{13}C-NMR ppm (150 MHz, $CDCl_3$): 150.6 (C-1), 107.3 (C-1a), 121.6 (C-1b), 146.1 (C-2), 101.7 (C-3), 134.6 (C-3a), 123.0 (C-4), 143.8 (C-5), 146.0 (C-6a), 180.3 (C-7), 124.9 (C-7a), 108.6 (C-8), 148.5 (C-9), 152.9 (C-10), 107.8 (C-11), 126.8 (C-11a), 55.1 (9-OCH_3), 55.3 (10-OCH_3), 101.4 (O-CH_2-O).

3.7.1.2 *Phoebe grandis* (bark) Constituents – KL 4994

β-sitosterol (59)

Fractions 1-5 of CC which appeared as whitish oily fractions were further subjected to small column chromatography. Fractions 8-11 afforded β-sitosterol, **59** (7.0 mg, 0.53

%) as white crystals with m.p 137°C, [(Hill *et al*., 1991) m.p 136-137°C] and R_f value of 0.6 in chloroform. (Refer Scheme 3.4)

Physical data:

$C_{29}H_{50}O$, colourless needles, $[\alpha]^{25}_D$ = -36.6°(c= 0.15, $CHCl_3$). Yield: 0.53 %

m.p. : 137°C

UV MeOH λ_{max} nm (log ε): 251 nm

IR ν_{max} cm^{-1} ($CHCl_3$): 3432, 2936, 1637 and 1460.

HREIMS $[M+H]^+$ m/z : 415.7750 (calcd for $C_{29}H_{51}O$, 415.7180)

^{1}H-NMR δ (600 MHz, $CDCl_3$, *J*/Hz): *δ* 5.33 (*m*, 1H, H-6), 3.50 (*tdd*, 1H, *J*=4.5, H-3), 0.99 (*s*, 3H, Me-27), 0.90 (*d*, 3H, *J*=6.5, Me-21), 0.82 (*t*, 3H, *J*=7.4, Me-29), 0.81 (*s*, 3H, Me-19), 0.79 (*d*, 3H, *J*=6.8, Me-26), 0.66 (*s*, 3H, Me-18) and other protons.

^{13}C-NMR ppm (150 MHz, $CDCl_3$): 140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.9 (C-24), 42.3 (C-4, C-13), 39.8 (C-12), 37.3 (C-1), 36.5 (C-20), 36.2(C-10), 34.0 (C-22), 31.9 (C-7, C-8), 31.7 (C-2), 29.2 (C-25), 28.2 (C-16), 26.1 (C-23), 24.3 (C-15), 23.1 (C-28), 21.1 (C-11), 19.8 (C-19), 19.4 (C-27), 19.0 (C-26), 18.8 (C-21), 12.0 (C-29), 11.9 (C-18).

Stigmasterol (60)

Fractions of 12-17 of CC were combined and separated using small gravity column chromatography (silica type : Sephadex LH 20) eluted with solvent mixture of n-hexane/ ethyl acetate ratio 9 : 1 to give 25 fractions. Fractions 7-16 obtained from the separation afforded **60**, stigmasterol (6.2 mg, 0.47 %) as needle crystals with m.p 167 - 168°C [(Schwartz & Wall, 1955) m.p 166-168°C)] and R_f value of 0.3 in chloroform. (Refer Scheme 3.4)

Physical data:

$C_{29}H_{48}O$, white powder, $[\alpha]^{25}_D$ = -20.6° (c= 0.15, $CHCl_3$). Yield: 0.47 %

m.p. : 167-168°C

UV MeOH λ_{max} nm (log ε): 257 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3351 (br), 2934, 1656, 1459, 1379, 1093, 1049, 970, 841, 747.

EIMS m/z (% intensity): 412 ([M+], 59.72), 394 (5.48), 379 (8.70), 351 (17.77), 300 (26.94), 271 (40.90), 255 (52.94), 213 (36.51), 159 (55.91), 133 (57.83), 119 (45.21), 105 (65.01), 95 (63.98), 81 (96.32).

^{1}H-NMR δ (500 MHz, $CDCl_3$, *J*/Hz):δ 5.14 (*dd*, *J*=15.2, 8.5, 1H, H-22), 5.02 (*dd*, *J*=15.2, 8.5, 1H, H-23), 5.36 (*m*, 1H, H-6), 3.52 (*tdd*, 1H, *J*=4.5, 4.2, 3.8, H-3), 2.40 – 0.67 (other protons).

^{13}C-NMR ppm (125 MHz, $CDCl_3$): δ 140.7 (C-5), 138.3 (C-6), 138.1 (C-22), 129.2 (C-23), 71.8 (C-3), 56.9 (C-14), 55.9 (C-17), 51.3 (C-24), 50.1 (C-9), 42.3 (C-4), 42.2 (C-13), 40.6 (C-20), 39.7 (C-12), 37.2 (C-1), 36.5 (C-10), 31.9 (C-25), 31.9 (C-8), 31.9 (C-7), 31.6 (C-2), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-26), 21.1 (C-21), 21.0 (C-11), 19.4 (C-19), 19.0 (C-27), 12.3 (C-29) and 12.0 (C-18).

Boldine (5)

Fractions of 190 - 226 of CC were combined and separated using small gravity column chromatography. Microcolumn was packed with silica gel Silica Gel 60F, 230 – 400 Mesh ASTM (Merck 7734) eluted with solvent mixture of DCM : MeOH ratio 9:1 to give 15 fractions. Fractions 5 – 10 obtained from the separation, and further purification by preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1, 97 : 3, 95 : 5) afforded **5**, boldine (5.5 mg, 0.14 %) as brownish amorphous solid with m.p 159-161°C [161°C reported from (Guinaudeau *et al.,* 1975)] and R_f value of 0.5 in dichloromethane. (Refer Scheme 3.5)

Physical data:

$C_{19}H_{21}O_4N$, brownish amorphous solid, R_f 0.5; $[\alpha]_D^{13}$ = +111°(c =0.5, C_2H_5OH) Yield: 0.14 %

m.p. : 159-161˚C.

UV MeOH λ_{max} nm (log ε): 220, 282 and 303 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3345 (OH), 2920 (CH), 1725, 1670, 1468, 1277, 1262, 909 and 733.

EIMS m/z (% intensity): *m/z* 328.00 (100 %) $[M+1]^+$, 265.08 (90 %).

^{1}H-NMR δ (400 MHz, $CDCl_3$): 7.87 (*s*, 1H, H-11), 6.83 (*s*, 1H, H-8), 6.64 (*s*, 1H, H-3), 3.90 (*s*, 3H, 10-OCH_3), 3.59 (*s*, 3H, 1-OCH_3), 2.54 (*s*, 3H, NCH_3), 2.85-3.10 (a complex pattern, C-4, 2H; C5, 2H; C-6a, 1H; C-7, 2H).

^{13}C-NMR ppm (100 MHz, $CDCl_3$): 148.1(C-2), 145.7 (C-10), 145.2 (C-9), 142.2 (C-1), 130.4 (C-7a), 130.1 (C-3a), 126.8 (C-1a), 126.0 (C-1b), 123.8 (C-11a), 114.3 (C-8), 113.4 (C-3), 110.3 (C-11), 62.7 (C-6a), 60.4 (C-1-OMe), 56.3 (C-10-OMe), 53.5 (C-5), 44.1 (NCH_3), 34.3 (C-7), 29.0 (C-4).

N-methyllaurotetanine (51)

Fractions 15 – 20 were combined and the resulted compounds were rechromatographed over a silica gel column, (Silica Gel 60F, 230 – 400 Mesh ASTM (Merck 7734)) eluted with solvent mixture of DCM: MeOH ratio 85 : 15 to give 40 fractions. Fractions 30 – 35 obtained from the separation, was purified by preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 97 : 3) to yield **51,** N-methyllaurotetanine (8 mg, 0.2 %). (Refer Scheme 3.5)

Physical data:

$C_{20}H_{23}NO_4$, yellow amorphous solid, $[\alpha]_D^{25}$ = +111° (c=1.0, MeOH). Yield: 0.2 %

m.p. : 237-238˚C

UV MeOH λ_{max} nm (log ε): 215, 283 and 305 nm.

IR ν_{max} cm^{-1}($CHCl_3$): 3390, 2935, 2359, 1591, 1444, 1318, 1253, 1111, 1085 and 909 cm^{-1}.

HREIMS m/z: $[M+H]^+$ at m/z 342.1716 (calcd for $C_{20}H_{24}NO_4$ = 342.1705)

^{1}H-NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.58 (*s*, 1H, H-3), 3.15(*dd*, *J*= 4.3, 15.5, H-4β), 2.66 (*dd*, *J*= 4.3, 15.5, H-4α), 3.06 (*dd*, *J*= 6.4, 12.4, H-5β), 2.48 (*dd*, *J*= 6.4, 12.4, H-5α), 2.99 (*m*, 1H, H-6a), 2.95 (*d*, *J*=4.1, H-7β) 2.51 (*d*, *J*=4.1, H-7α), 6.85 (*s*,1H, H-8), 8.04 (*s*,1H, H-11), 3.62 (*s*, 3H, OMe-1), 3.86 (*s*, 3H, OMe-2), 3.89 (*s*, 3H, OMe-10), 2.54 (*s*, 3H, NMe).

^{13}C-NMR ppm (100 MHz, $CDCl_3$): 152.0 (C-2), 145.3 (C-10), 144.9 (C-9), 144.2 (C-1), 130.2 (C-7a), 129.0 (C-3a), 127.2 (C-1b), 127.1(C-1a), 124.0 (C-11a), 114.0 (C-8), 111.2 (C-11), 110.3 (C-3), 62.6 (C-6a), 60.2 (C-1-OMe), 56.1 (C-10-OMe), 55.9 (C-2-OMe), 53.3 (C-5), 44.0 (NMe), 34.3 (C-7), 29.3 (C-4).

Reticuline (61)

Fractions 30 – 65 obtained from fractionation of dichloromethane extract of bark of *Phoebe grandis* (KL 4994), the fractions was evaporated to dryness. The dark brown solid obtained was further rechromatographed over a silica gel column, (Silica Gel 60F, 230 – 400 Mesh, ASTM (Merck 7734)) eluted with solvent mixture of DCM: MeOH ratio 95: 5 to give another 20 fractions. Fractions 11 – 19 obtained from the separation, was purified by preparative TLC (Silica gel $60F_{254}$, developed in CH_2Cl_2: MeOH; 99: 1 and 98: 2; saturated with NH_3 vapours) afforded **61**, reticuline (10.0 mg, 0.25 %). (Refer Scheme 3.5)

Physical data:

$C_{19}H_{23}NO_4$, brownish amorphous solid, $[\alpha]_D^{25}$ = + 0.003° (c=0.5, MeOH), Yield: 0.25 %

m.p. : 200 - 202 °C.

UV MeOH λ_{max} nm (log ε): 235 and 283 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3392 cm^{-1} and 2918 cm^{-1}.

LCMS -Q TOF m/z: 330.1695 $[M+H]^+$, (calcd for $C_{19}H_{24}NO_4$, 330.1661).

^{1}H-NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 3.66 (1H, *t*, *J*=6.0, H-1), 2.80-3.20 (2H, *m*, H-3), 2.50-2.85 (2H, *m*, H-3), 6.50 (*s*, 1H, H-5), 6.30 (*s*, 1H, H-8), 2.75-3.05 (2H, *m*, H-1α), 6.77 (1H, *d*, *Jm*=1.9, H-2'), 6.68 (1H, *d*, *J*=8.0, H-5'), 6.51 (1H, *dd*, *Jo*=8.0, *Jm*=1.7, H-6'), 3.84 (3H, *s*, 6-OCH_3), 3.80 (3H, *s*, 4'-OCH_3), 2.44 (3H, *s*, N-CH_3).

^{13}C-NMR ppm (100 MHz, $CDCl_3$): 145.5 (C-4'), 145.4 (C-3'), 145.3 (C-6), 143.5 (C-7), 132.8 (C-1'), 129.7 (C-4a), 124.7 (C-8a), 121.0 (C-6'), 115.8 (C-2'), 114.0 (C-8), 110.7 (C-5'), 110.6 (C-5), 64.4 (C-1), 55.9 (C-4'), 55.8 (6-OCH_3), 46.4 (C-3), 42.1 (N-CH_3), 40.8 (C-1α), 24.7 (C-4).

Laurolitsine (6)

Fractions 227 – 235 of the extracts from first column chromatography eluted with dichloromethane: methanol (60 : 40) were combined and subjected to preparative TLC on Si gel using dichloromethane : methanol (95 : 5). This afforded **6**, laurolitsine (7.0 mg, 0.18 %). (Refer Scheme 3.5)

Physical data:

$C_{18}H_{19}NO_4$, brownish amorphous solid. R_f 0.3; $[\alpha]_D^{25}$ = + 6.77° (c=0.5, MeOH). Yield: 0.18 %

m.p. : 113-115°C

UV MeOH λ_{max} nm (log ε): 282 nm and 307 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3340, 2025, 1590, 1521, 1469, 1260, 1090, 1021 and 758 cm^{-1}. A broad band between 3500 and 2500 cm^{-1} is due to the presence of OH and NH functional groups.

LCMS -Q TOF m/z: 313.1397 $[M+H]^+$, (calcd for $C_{18}H_{20}NO_4$, 314.1314).

^{1}H-NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.60 (1H, *s*, H-3), 2.61 (1H, *m*, H-4α), 2.91 (1H, *m*, H-4β), 2.94 (1H, *m*, H-5α), 3.31 (1H, *m*, H-5β), 3.75 (1H, *dd*, *J*=13.7, *J*=4.1, H-6a), 2.70 – 2.60 (2H, *m*, H-7), 6.73 (1H, *s*, H-8), 3.59 (3H, *s*, 1-OCH_3), 3.87 (3H, *s*, 10-OCH_3), 7.95 (1H, *s*, H-11).

^{13}C-NMR ppm (100 MHz, $CDCl_3$): 149.5 (C-2), 146.8 (C-10), 146.3 (C-9), 142.3 (C-1), 127.4 (C-1b), 126.5 (C-1a), 123.8 (C-11a), 114.5 (C-8), 114.4 (C-3), 110.6 (C-11), 60.2 (1-OCH_3), 56.3 (10-OCH_3), 53.6 (C-6a), 43.0 (C-5), 36.4 (C-7), 29.0 (C-4).

3.7.1.3 *Phoebe tavoyana* (leaves) Alkaloids – KL 5225

Laetanine (62)

The combined fractions 15 – 20 was carried out by chromatography on a small column with CH_2Cl_2 containing increasing amount of MeOH (85 : 15), to get fraction 30 – 35. Further purification by preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1 and 98 : 2) yielded **62**, laetanine (8.0 mg, 0.1 %). (Refer Scheme 3.6)

Physical data:

$C_{18}H_{19}NO_4$, dark brown amorphous solid, $[\alpha]_D^{25}$ = + 105° (c=0.4, MeOH), Yield: 0.1 %

m.p. : 226-228°C

UV MeOH λ_{max} nm (log ε): 284 nm and 304 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3350, 2920, 1566, 1250, 1210, 1078 and 754 cm^{-1}. A broad band appeared between 3200 - 3400 cm^{-1} indicated the presence of hydroxyl and NH group.

LCMS Q- TOF *m/z*: 314.1313 $[M+H]^+$, (calcd for $C_{18}H_{20}NO_4$, 314.1314).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.58 (1H, *s*, H-3), 2.66 (1H, *m*, H-4α), 2.98 (1H, *m*, H-4β), 2.95 (1H, *m*, H-5α), 3.30 (1H, *m*, H-5β), 3.80 (1H, *dd*, *J*=14.2, *J*=4.5, H-6a), 2.60 (1H, *dd*, *J*=13.7, *J*=4.5, H-7α), 2.75 (1H, *dd*, *J*=13.7, *J*=4.5, H-7β), 6.75 (1H, *s*, H-8), 3.55 (3H, *s*, 1-OCH_3), 3.80 (3H, *s*, 9-OCH_3), 7.89 (1H, *s*, H-11).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 149.1 (C-2), 146.2 (C-9), 145.5 (C-10), 142.7 (C-1), 128.6 (C-7a), 128.4 (C-3a), 126.2 (C-1a), 124.9 (C-1b), 123.3 (C-11a), 114.5 (C-8), 113.9 (C-3), 110.9 (C-11), 60.1 (1-OCH_3), 56.1 (9-OCH_3), 53.4 (C-6a), 42.4 (C-5), 35.2 (C-7), 27.4 (C-4).

Roemerine or *N*-methyl-anonaine (20)

Fraction No. 40 was rechromatographed on Si gel column using $CHCl_3$: MeOH for gradient elution, to get subfractions 5 – 15 (95 : 5). Then, followed by purification on preparative TLC using $CHCl_3$: MeOH (9 : 1) to afford **20**, roemerine or *N*-methyl-anonaine (5.0 mg, 0.063 %). (Refer Scheme 3.6)

Physical data:

$C_{18}H_{17}NO_2$, white amorphous solid, $[\alpha]_D^{25}$ = - 0.180° (c= 0.5, C_2H_6OH). Yield: 0.063 %

m.p. : 100-103°C.

UV MeOH λ_{max} nm (log ε): 234, 264, 273, 285, 293 and 320 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 2813, 1588, 1521, 1450, 1410, 1223, 1039 and 953 cm^{-1}.

LCMS Triple TOF *m/z*: 280.1257 $[M+H]^+$, (calcd. for $C_{18}H_{18}NO_2$, 280.1259).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 8.04 (1H, *d*, *J*=7.0, H-11), 7.20 – 7.32 (3H, *m*, H-8, H-9, H-10), 6.55 (1H, *s*, H-3), 6.08 and 5.93 (2H, *d*, *J*=1.4, -OCH_2O-), 3.49 (1H, *d*, *J*=13.7, H-6a), 3.24 (1H, *d*, *J*=4.2, H-5β), 3.19 (1H, *d*, *J*=4.4, H-5α), 2.90 (1H, *d*, *J*=10.2, H-4β), 2.70 (1H, *d*, *J*=10.2, H-4α), 2.73 (1H, *m*, H-7β), 2.71 (1H, *m*, H-7α), 2.67 (3H, *s*, N-CH_3).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 146.6 (C-2), 142.5 (C-1), 135.1 (C-7a), 130.9 (C-11a), 128.1 (C-3a), 127.4 (C-11), 126.9 (C-10), 126.7 (C-9), 126.5 (C-8), 126.3 (C-1b), 100.6 (OCH_2O), 61.9 (C-6a), 53.4 (C-5), 43.6 (N-CH_3), 34.3 (C-7), 28.9 (C-4).

Laurolitsine or norboldine (6)

Fraction 45 (eluting with 92 % DCM : 8 % MeOH) from first column chromatography was subjected to mini column chromatography to yield 10 fractions. Fractions 3 – 10 (eluting with 90 % DCM : 10 % MeOH) showed similar spot on TLC plate using DCM : MeOH (98 : 2) and were combined to give **6**, laurolitsine (6.5 mg, 0.08 %). (Refer Scheme 3.6)

Physical data:

$C_{18}H_{19}NO_4$, brownish amorphous solid. R_f 0.3; $[\alpha]_D^{25}$ = + 6.77°(c=0.167, MeOH). Yield: 0.08 %

Spectral data were identical to laurolitsine (**6**) isolated from dichloromethane extract of bark of *Phoebe grandis*, previously discussed in section **3.7.1.2.** Please refer **6** (page 77).

Tavoyanine A (63)

Fractions of 48 – 50 were combined from first column chromatography. It was a mixture of compound **63** and **64** then, further separated via column chromatography to give subfractions 5 – 10 yellowish supernatant (88.0 mg). These fractions were pooled and evaluated according to TLC analysis using DCM : MeOH (99 : 1, v/v three times) yielding a new pure compound of **63**, tavoyanine A (8.0 mg, 0.1 %). It was isolated for the first time as natural compounds. (Refer Scheme 3.6)

Physical data:

$C_{21}H_{25}NO_5$, dark brown amorphous solid, $[\alpha]_D^{25}$ = + 0.016° (c= 0.5, CH_3OH). Yield: 0.1 %

UV MeOH λ_{max} nm (log ε): 283 nm and 305 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3360, 2928, 2825, 1590, 1510, 1465, 1258, 1080, 995 and 750 cm^{-1}.

LCMS-ESI *m/z*: 372.1776 $[M+H]^+$, (calcd. for $C_{21}H_{26}NO_5$, 372.1811).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 7.80 (1H, *s*, H-11), 6.76 (1H, *s*, H-8), 6.58 (1H, *s*, H-3), 3.88 (1H, *m*, H-13), 3.17 (1H, *dd*, *J*=13.7, *J*=3.2, H-6a), 3.11 (1H, *dd*, *J*=4.1, *J*=11.3, H-5β), 2.94 (1H, *dd*, *J*=3.7, *J*=12.0, H-7β), 2.70 (1H, *dd*, *J*=2.7, *J*=12.6, H-12β), 2.60 (1H, *d*, *J*=4.1, H-4β), 2.56 (1H, *d*, *J*=16.0, H-4α), 2.42 (1H, *dd*, *J*=3.7, *J*=12.0, H-7α), 2.38 (1H, *dd*, *J*=4.1, *J*=11.3, H-5α), 2.18 (1H, *dd*, *J*=2.7, *J*=12.6, H-12α), 1.12 (3H, *d*, 6.4, H-14), 3.50 (3H, *s*, 1-OCH_3), 3.85 (3H, *s*, 10-OCH_3).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 148.2 (C-2), 145.8 (C-10), 145.3 (C-9), 142.2 (C-1), 130.4 (C-3a), 130.2 (C-7a), 127.3 (C-1b), 126.6 (C-1a), 123.7 (C-11a), 114.3 (C-8), 113.3 (C-3), 110.3 (C-11), 62.9 (C-13), 61.5 (C-12), 60.44 (C-6a), 60.40 (1-OCH_3), 56.4 (10-OCH_3), 49.4 (C-5), 34.9 (C-7), 29.4 (C-4), 19.8 (C14).

Tavoyanine B (64)

Combined fractions of 48 – 50 were obtained from first column chromatography. It was a mixture of compound **63** and **64** then, further separated via column chromatography to give subfractions 5 – 10 of yellowish supernatant (88 mg). These fractions were pooled and evaluated according to TLC analysis using DCM : MeOH (98 : 2, v/v three times) yielding a new pure compound of **64**, tavoyanine B (7.0 mg, 0.09 %). This compound also isolated as a new natural compounds for the first time from plant. (Refer Scheme 3.6)

Physical data:

$C_{21}H_{25}NO_5$, brownish amorphous solid, $[\alpha]_D^{25}$ = - 0.03° (c=0.5, CH_3OH).Yield: 0.09 %

UV MeOH λ_{max} nm (log ε): 293 nm and 304 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 3300, 2935, 2838, 1568, 1453, 1260, 1084, and 750 cm^{-1}.

LCMS-ESI *m/z*: 372.1804 $[M+H]^+$, (calcd. for $C_{21}H_{26}NO_5$, 372.1811).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 7.88 (1H, *s*, H-11), 6.82 (1H, *s*, H-8), 6.65 (1H, *s*, H-3), 3.85 (1H, *m*, H-13), 3.40 (1H, *dd*, *J*=3.9, 13.9, H-6a), 3.06 (1H, *m*, H-5β), 3.00 (1H, *m*, H-4β), 2.88 (1H, *dd*, *J*=3.9, 13.6, H-12β), 2.83 (1H, *m*, H-5α), 2.79 (1H, *m*, H-7β), 2.66 (1H, *m*, H-4α), 2.62 (1H, *m*, H-7α), 2.36 (1H, *dd*, *J*=9.3, 13.6, H-12α), 1.20 (3H, *d*, *J*=6.1, H-14), 3.59 (3H, *s*, 1-OCH_3), 3.92 (3H, *s*, 10-OCH_3).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 148.1 (C-2), 145.7 (C-10), 145.2 (C-9), 142.1 (C-1), 130.3 (C-7a), 130.2 (C-3a), 127.2 (C-1b), 126.2 (C-1a), 123.6 (C-11a), 114.3 (C-8), 113.3 (C-3), 110.1 (C-11), 66.0 (C-13), 63.3 (C-12), 61.4 (C-6a), 60.4 (1-OCH_3), 56.3 (10-OCH_3), 52.8 (C-5), 35.3 (C-7), 29.0 (C-4), 20.6 (C14).

Boldine (5)

Fraction 50 from the first column chromatography (eluting with 90 % DCM: 10 % MeOH) was rechromatographed using small gravity column chromatography. Microcolumn was packed with silica gel Silica Gel 60F, 230 – 400 Mesh ASTM (Merck 7734) eluted with solvent mixture of DCM : MeOH ratio 95 : 5 to give 15 fractions. Subfractions 7 – 11 obtained from the separation, and further purification via preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 97 : 3, saturated with NH_3) afforded brownish amorphous solid **5**, boldine (5.5 mg, 0.07 %). (Refer Scheme 3.6)

Physical data:

$C_{19}H_{21}O_4N$, brownish amorphous solid, R_f 0.45; $[\alpha]_D^{13}$ = +111° (c =1.03, C_2H_5OH)

Yield: 0.07 %

Spectral data were identical to boldine (**5**) isolated from dichloromethane extract of bark of *Phoebe grandis*, previously discussed in section **3.7.1.2**. Please refer **5** (page 74-75).

Sebiferine (22)

Fraction No. 55 (eluting with 85 % DCM : 15 % MeOH) was repeatedly chromatographed on Si gel column using (90 % DCM : 10 % MeOH) with gradient elution to produce 15 series of subfractions. Further purification via preparative TLC using 98 % DCM : 2 % MeOH yielded **22**, sebiferine (5.0 mg, 0.06 %). (Refer Scheme 3.6)

Physical data:

$C_{20}H_{23}NO_4$, pale yellow amorphous solid, $[\alpha]_D^{25}$ = + 1.38° (c = 0.5, $CHCl_3$). Yield: 0.06 %

m.p. : 111-113˚C.

UV MeOH λ_{max} nm (log ε): 209, 238 and 280 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 1660, 1639 and 1615 cm^{-1}.

LCMS-ESI *m/z*: 342.1692 $[M+H]^+$ (calcd. mass for $C_{20}H_{24}NO_4$, 342.1627).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.77 (1H, *s*, H-4), 6.58 (1H, *s*, H-1), 6.32 (1H, *s*, H-5), 6.22 (1H, *s*, H-8), 3.60 (1H, *d*, *J*=6.1, H-9), 3.32 (1H, *dd*, *J*=6.4, *J*=17.2, H-10β), 2.98 (1H, *dd*, *J*=6.4, *J*=17.2, H-10α), 2.46 (2H, *m*, H-16), 1.82 (1H, *m*, H-15), 3.82 (3H, *s*, 2-OCH_3), 3.78 (3H, *s*, 3-OCH_3), 3.65 (3H, *s*, 6-OCH_3), 2.40 (3H, *s*, N-CH_3).

^{13}C NMR ppm (100 MHz, $CDCl_3$): 180.3 (C-7), 161.5 (C-14), 151.7 (C-6), 148.7 (C-3), 148.5 (C-2), 130.4 (C-12), 128.1 (C-11), 122.1 (C-8), 118.6 (C-5), 110.0 (C-1), 108.3 (C-4), 60.2 (C-9), 55.8 (2-OCH_3), 55.5 (3-OCH_3), 55.4 (6-OCH_3), 45.1 (C-16), 41.7 (NMe), 41.1 (C-13), 40.6 (C-15), 31.9 (C10).

3.7.1.4 *Actinodaphne sesquipedalis* (leaves) Constituents – HIR 011

β-sitosterol (59)

Fractions 2 – 4 of first column chromatography were combined and rechromatographed using small gravity Sephadex LH-20 column chromatography eluted with solvent mixture of n-hexane / ethyl acetate ratio 50 : 50 to give 20 subfractions. Subfractions 3 – 18 obtained from the separation appeared as whitish oily fractions. It was further purified by washing with MeOH and afforded **59**, as white needle crystals (5.5 mg, 0.06 %). (Refer Scheme 3.7)

Physical data:

$C_{29}H_{50}O$, colourless needles, $[\alpha]^{25}_{D}$ = -36.6° (c= 0.15, $CHCl_3$). Yield: 0.06 %

Spectral data were identical to β-sitosterol (**59**) that previously isolated from hexane extract of bark of *Phoebe grandis*. It was described earlier in section **3.7.1.2** (refer page 72-73).

Dicentrine (52)

Fraction 9 was obtained from fractionation of hexane extract and evaporated to dryness. The dark brown solid obtained was further rechromatographed over a silica gel column, (Silica Gel 60F, 230 – 400 Mesh ASTM (Merck 7734)) eluted with solvent mixture of n-hexane : EA ratio 80 : 20 to give 10 fractions. Subfractions 5 – 10 obtained from the separation, was purified by preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1) to yield **52**, dicentrine (4.0 mg, 0.04 %). (Refer Scheme 3.7)

Physical data:

$C_{20}H_{21}NO_4$, crystallized as a colourless prism, $[\alpha]^{25}_{D}$ = +65° (c= 0.65, $CHCl_3$). Yield: 0.04 %

m.p. : 167-169°C.

UV MeOH λ_{max} nm (log ε): 219, 281 and 305 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 940, 1095 (methylenedioxy), 1268 (methoxyl), 1344 – 1606 cm^{-1} (aromatic C=C stretching), 2790 and 2916 cm^{-1} (CH-aromatic).

LCMS-QTOF m/z: 340.1534 $[M+H]^+$, (calcd for $C_{20}H_{22}NO_4$, 340.1471).

^{1}H-NMR δ (600 MHz, $CDCl_3$, *J*/Hz): 7.63 (1H, *s*, H-11), 6.75 (1H, *s*, H-8), 6.48 (1H, *s*, H-3), 6.04 and 5.89 (1H each, *d*, *J*=1.32, O-CH_2-O), 3.88 (3H, *s*, 9-OCH_3), 3.87 (3H, *s*, 10-OCH_3), 3.14 (1H, *m*, H-6a), 3.06 (*dd*, *J*=14.4,4.4, $H_{4\beta}$), 3.04 (*d*, *J*=4.3, $H_{7\beta}$), 3.03 (*dd*, *J*=12.4, 4.8, $H_{5\beta}$), 2.64 (*d*, *J*=4.3, $H_{7\alpha}$), 2.59 (*dd*, *J*=14.4, 4.4, $H_{4\alpha}$), 2.53 (3H, *s*, N-CH_3), 2.49 (*dd*, *J*=12.4, 4.8, $H_{5\alpha}$).

^{13}C-NMR ppm (150 MHz, $CDCl_3$): 148.3 (C-9), 147.7 (C-2), 146.7 (C-10), 141.8 (C-1), 128.3 (C-7a), 126.6 (C-3a), 126.2 (C-1b), 123.6 (C-11a), 116.6 (C-1a), 111.3 (C-8), 110.6 (C-11), 106.8 (C-3), 100.6 (O-CH_2-O), 62.3 (C-6a), 56.1 (10-OCH_3), 55.9 (9-OCH_3), 53.5 (C-5), 43.7 (N-CH_3), 34.1 (C-7), 29.0 (C-4).

N-methyllaurotetanine (51)

Fraction 10 from the first column chromatography (eluting with 90 % n-hexane : 10 % ethyl acetate) was passed through a small Sephadex LH-20 column eluted with solvent mixture of n-hexane : EA ratio 40 : 60 to give 15 subfractions with 50 mL each. The eluates of subfractions 2-11 were monitored on TLC performed on precoated aluminium sheets (Silica Gel 60 F_{254}, 0.25 mm, CH_2Cl_2 : MeOH; 97 : 3, saturated with NH_3) were observed under UV (254/365nm) light and then sprayed with Dragendorffs reagent or H_2SO_4. The fractions were combined on the basis of similar TLC profiles and afforded yellow amorphous solid **51**, N-methyllaurotetanine (6.0 mg, 0.07 %). (Refer Scheme 3.7)

Physical data:

$C_{20}H_{23}NO_4$, yellow amorphous solid, $[\alpha]_D^{25}$ = +111° (c=1.0, MeOH). Yield: 0.07 %

Spectral data were identical to N-methyllaurotetanine (**51**) isolated from dichloromethane extract of bark of *Phoebe grandis*, previously discussed in section **3.7.1.2.** Please refer (page 75-76).

Stigmasterol (60)

Fraction No. 16 from the first column chromatography (eluting with 90 % n-hexane : 10 % EA). The eluates with the white powder was repeatedly chromatographed on Sephadex LH-20 column using (70 % n-hexane : 30 % EA) to produce 20 series of subfractions. Then, the combined fractions 3 – 18 was further purified by washing with MeOH and recryatallised with pet. ether and MeOH to yield compound **60**, stigmasterol (5 mg, 0.06 %). (Refer Scheme 3.7)

Physical data:

$C_{29}H_{48}O$, white powder, $[\alpha]^{25}_{D}$ = -20.6° (c= 0.15, $CHCl_3$). Yield: 0.06 %

Spectral data were identical to stigmasterol (**60**) isolated from hexane extract of bark of *Phoebe grandis*, previously discussed in section **3.7.1.2**. Please refer (page 73-74).

β-sitosterol (59)

Fractions 1 – 7 of first column chromatography (Sephadex LH-20, eluted with MeOH) were combined and rechromatographed using Sephadex LH-20 small gravity column (n-hexane : MeOH, ratio 40 : 60) to produce 15 subfractions. Fractions were evaluated and pooled according to TLC analysis. Fractions 10 – 14 were evaporated to dryness and further purified by washing with MeOH and recryatallised with pet. ether and MeOH to yield compound colourless needles, **59**, β-sitosterol (7.0 mg, 0.07 %). (Refer Scheme 3.8)

Physical data:

$C_{29}H_{50}O$, colourless needles, $[\alpha]^{25}_{D}$ = -36.6° (c= 0.15, $CHCl_3$). Yield: 0.07 %

Spectral data were identical to β-sitosterol (**59**) that previously isolated from hexane extract of bark of *Phoebe grandis* and hexane extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.2.** Please refer (page 72-73).

Dicentrine (52)

Fractions 8 – 12 of first column chromatography (Sephadex LH-20 eluated with MeOH) were combined and evaporated to dryness. Then, purification via preparative TLC with the solvent system DCM : MeOH (98 : 2, saturated with NH_3) gives a very broad band on TLC when observed under UV (254/365nm) light and then sprayed with Dragendorffs reagent shown to contain alkaloids with the orange spot. The greenish-blue band was scraped and washed with DCM. Evaporation of the solvent yielded a yellow semisolid was subjected to mini column chromatography Sephadex LH-20 using n-hexane to give another 10 fractions. The subfractions 5 – 8 were combined on the basis of similar TLC profiles and afforded yellow amorphous solid **52**, dicentrine (10.0 mg, 0.1 %). (Refer Scheme 3.8)

Physical data:

$C_{20}H_{21}NO_4$, crystallised as a colourless prism, $[\alpha]^{25}_D$ = +65° (c= 0.65, $CHCl_3$). Yield: 0.1 %

Spectral data were identical to dicentrine (**52**) that previously isolated from hexane extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.4**. Please refer (page 84-85).

Dicentrinone (58)

Fractions 13 – 20 were obtained from fractionation of methanol extract were evaporated to dryness. The dark brown solid obtained was further rechromatographed over a silica gel column, (Silica Gel 60F, 230 – 400 Mesh ASTM (Merck 7734)) eluted with solvent

mixture of n-hexane : DCM : MeOH with the ratio 5 : 85 : 10 to give 15 subfractions. Subfractions 9 – 14 obtained from the separation, was purified by preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 98 : 2) to yield yellow fine needles, **58**, dicentrinone (5.5 mg, 0.055 %). (Refer Scheme 3.8)

Physical data:

$C_{19}H_{13}NO_5$, yellow fine needles. Yield: 0.055 %

Spectral data were identical to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis*, previously discussed in section **3.7.1.1.** Please refer (page 72).

Boldine (5)

Fractions 21 – 28 were combined fractions from the Sephadex LH-20 column eluted with 100 % MeOH were evaporated to dryness. The dark brown solid obtained was further rechromatographed on Sephadex LH-20 column using (50 % n-hexane: 50 % MeOH) to produce 10 series of subfractions. Finally, subfractions 5 – 8 were further separated and purified via preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1) to yield **5**, boldine (5.0 mg, 0.05 %). (Refer Scheme 3.8)

Physical data:

$C_{19}H_{21}O_4N$, brownish amorphous solid, R_f 0.5; $[\alpha]_D^{13}$ = +111 (c =0.5, C_2H_5OH),Yield: 0.05 %

Spectral data were identical as boldine (**5**) isolated from dichloromethane extract of bark of *Phoebe grandis*, previously discussed in section **3.7.1.2.** Please refer (page 74-75).

Norisocorydine (65)

Fractions 29 – 37 eluted from 100 % MeOH of Sephadex LH-20 column were further separated and purified by silica gel column chromatography with DCM : MeOH (85 :

15) to yield 25 subfractions (50 mL eluate each). Subfractions 15 – 21 obtained from the separation, was purified twice via preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1, 97 : 3) to yield **65**, norisocorydine (7.5 mg, 0.075 %). (Refer Scheme 3.8)

Physical data:

$C_{19}H_{21}NO_4$, as a brownish amorphous solid, $[\alpha]_D^{25}$ = + 150.8° (c=0.08, CH_5OH), Yield: 0.075 %

m.p. : 201-203 °C.

UV EtOH λ_{max} nm (log ε): 223, 269 and 308 nm.

IR ν_{max} cm^{-1} (KBr): a broad band between 3400 cm^{-1} and 2960 cm^{-1} (OH and NH), 2960 and 2850 cm^{-1} (CH-aromatic), 1285 (methoxyl).

LCMS-ESI *m/z*: 328.1538 $[M+H]^+$, (calcd. for $C_{19}H_{22}NO_4$, 328.1549).

^{1}H NMR δ (400 MHz, $CDCl_3$, *J*/Hz): 6.85 (*d*, *J*=8.2, H-9), 6.75 (*d*, *J*=8.2, H-8), 6.70 (*s*, 1H, H-3), 3.90 (*s*, 3H, OMe-10), 3.89 (*s*, 3H, OMe-2), 3.67 (*s*, 3H, OMe-1), 3.67 (*m*, 1H, H-6a), 3.43 (*m*, H-5β), 3.00 (*m*, H-4β), 2.92 (*m*, H-5α), 2.76 (*dd*, *J*=4.1, 13.3, H-7β), 2.70 (*m*, H-4α), 2.56 (*t*, *J*=13.3, H-7α).

^{13}C-NMR ppm (100 MHz, $CDCl_3$): 151.5 (C-2), 149.5 (C-10), 144.2 (C-11), 142.1 (C-1), 130.4 (C-3a), 130.6 (C-7a), 129.9 (C-1b), 125.8 (C-1a), 120.2 (C-11a), 119.3 (C-8), 111.8 (C-3), 110.9 (C-9), 62.3 (C-1-OMe), 56.7 (C-2-OMe), 56.5 (C-10-OMe), 54.2 (C-6a), 42.9 (C-5), 38.5 (C-7), 29.4 (C-4).

Laurolitsine or norboldine (6)

Fractions 38 – 50 were the last combined fraction from the Sephadex LH-20 column eluted with 100 % MeOH. The eluates with the yellow colour was repeatedly chromatographed on Sephadex LH-20 column using (50 % $CHCl_3$: 50 % MeOH) to produce 10 series of subfractions. Finally, subfractions 7 – 10 were further separated

and purified via preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1) to yield **6**, laurolitsine or norboldine (6.5 mg, 0.065 %). (Refer Scheme 3.8)

Physical data:

$C_{18}H_{19}NO_4$, brownish amorphous solid. $[\alpha]_D^{25}$ = + 6.77 (c=0.5, MeOH). Yield: 0.065 %

Spectral data were identical to laurolitsine (**6**) that isolated from dichloromethane extract of bark of *Phoebe grandis* and leaves of *Phoebe tavoyana*. It was described earlier in section **3.7.1.2.** Please refer (page 77-78).

3.7.1.5 *Actinodaphne sesquipedalis* (fruits) Constituents – HIR 011

Dicentrine (52)

Fraction 9 (3.0 g) of the first column which contains compound **52** and **58** was loaded on silica gel column, Sephadex LH 20 using n-hexane : ethyl acetate (60 : 40) to purify compounds **52** (62 mg, 8.86 %) from a less polar zone and **58** (6.3 mg), from a more polar zone, respectively. (Refer Scheme 3.9)

Physical data:

$C_{20}H_{21}NO_4$, crystallized as a colourless prism, $[\alpha]^{25}_D$ = +65° (c= 0.65, $CHCl_3$). Yield: 8.86 %

Spectral data were identical to dicentrine (**52**) that previously isolated from hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.4.** Please refer (page 84-85).

Liriodenine (19)

Fraction 5 (6.8 g) from the first column chromatography was loaded on silica gel and eluted with hexane : ethyl acetate (6 : 4) to give another 15 fractions. Fractions 7 – 14

obtained from the separation afforded solid yellow needles as compound **19**, liriodenine (3.0 mg, 0.04 %). (Refer Scheme 3.9)

Physical data:

$C_{17}H_9NO_3$, yellow needles. Yield: 0.04 %

m.p. : 280-282°C.

UV MeOH λ_{max} nm (log ε): 256, 281 and 334 nm.

IR ν_{max} cm^{-1} ($CHCl_3$): 860, 952, 1055 (methylenedioxy), 1636 (C=O), 2335, 2355 and 2970 cm^{-1} (CH-aromatic), 3580 (N-amine).

LCMS-QTOF m/z: 276.0653 $[M+H]^+$, (calcd for $C_{17}H_{10}NO_3$, 276.0582).

^{1}H-NMR δ (600 MHz, $CDCl_3$, *J*/Hz): 8.85 (1H, *d*, *J*=5.2, H-5), 8.58 *dd* (1H, *J*=8.0, 1.5, H-11), 8.54 (*dd*, *J*=8.0, 0.7, H-8), 7.72 (1H, *d*, *J*=5.2, H-4), 7.69 (1H, *dt*, *J*=8.0, 1.5, H-10), 7.54 (1H, *dt*, *J*=8.0, 0.7, H-9), 7.13 (1H, *s*, H-3), 6.34 (2H, *s*, O-CH_2-O).

^{13}C NMR ppm (150 MHz, $CDCl_3$): 182.4 (C-7), 151.7 (C-2), 147.9 (C-1), 145.4 (C-3a), 144.9 (C-5), 135.7 (C-6a), 133.9 (C-10), 132.9 (C-11a), 131.3 (C-7a), 128.8 (C-8), 128.5 (C-9), 127.3 (C-11), 124.2 (C-4), 123.2 (C-1b), 108.1 (C-1a), 103.2 (C-3), 102.5 (O-CH_2-O).

Dicentrinone (58)

Fraction 9 (3.0 g) of the first column which contains compound (**52**) and (**58**) was loaded on silica gel column, Sephadex LH 20 using hexane : ethyl acetate (60 : 40) and hexane : ethyl acetate (50 : 50) to purify the compound, from a more polar zone. Fractions 5 – 30 obtained from the separation afforded **58**, dicentrinone (6.3 mg, 0.09 %). (Refer Scheme 3.9)

Physical data:

$C_{19}H_{13}NO_5$, yellow fine needles. Yield: 0.09 %

Spectral data were identical to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis* and methanol extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.1.** Please refer (page 72).

β-sitosterol (59)

Fraction 12 (2.2 g) was subjected to column chromatography (Sephadex LH 20) and eluted with hexane : ethyl acetate (7 : 3) to give fractions 12 – 28. Then, the combined fractions 12 – 28 was further purified by washing with MeOH and recryatallised with pet. ether and MeOH to yield compound **59**, β-sitosterol (5 mg, 0.07 %). (Refer Scheme 3.9)

Physical data:

$C_{29}H_{50}O$, colourless needles, $[\alpha]^{25}_{D}$ = -36.6° (c= 0.15, $CHCl_3$). Yield: 0.07 %

Spectral data were identical as β-sitosterol (**59**) that previously isolated from hexane extract of bark of *Phoebe grandis* and hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.2.** Please refer (page 72-73).

Dicentrine (52)

Fractions 1 – 5 were subjected to column chromatography (Sephadex LH-20) and eluted with hexane to give 15 subfractions. Fractions 10 – 14 were evaluated and pooled according to TLC analysis. Further purification via preparative TLC (Silica gel $60F_{254}$, CH_2Cl_2 : MeOH; 99 : 1 and 98 : 2) yielded **52**, dicentrine (8.0 mg, 0.08 %). (Refer Scheme 3.10)

Physical data:

$C_{20}H_{21}NO_4$, crystallized as a colourless prism, $[\alpha]^{25}_{D}$ = +65° (c= 0.65, $CHCl_3$). Yield: 0.08 %

Spectral data were identical to dicentrine (**52**) that previously isolated from hexane and methanol extract of leaves of *Actinodaphne sesquipedalis;* and hexane extract of fruits of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.4.** Please refer (page 84-85).

β-sitosterol (59)

The combined fractions 6 – 10 was separated on silica Sephadex LH-20 column. Fractions 6 – 10 was purified via preparative TLC using 99 % DCM : 1 % MeOH and 98 % DCM : 2 % MeOH to afford the pure compound. Then, further purification by washing with MeOH and recrystallised with pet. ether and MeOH to yield compound **59**, β-sitosterol, (10.0 mg, 0.1 %). (Refer Scheme 3.10)

Physical data:

$C_{29}H_{50}O$, colourless needles, $[\alpha]^{25}_{D}$ = -36.6° (c= 0.15, $CHCl_3$). Yield: 0.1 %

Spectral data were identical to β-sitosterol (**59**) that previously isolated from hexane extract of bark of *Phoebe grandis*; hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*; and hexane extract of fruits of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.2**. Please refer (page 72-73).

Dicentrinone (58)

Fraction 11 - 15 from the big column was further purified on silica gel microcolumn eluted with increasing amount of MeOH in dichloromethane and hexane (Hex : DCM : MeOH; 5 : 85 : 10) yielding 10 subfractions of 50 mL each that were evaluated and pooled according to TLC analysis. Subfraction 5 - 8 was further rechromatographed using Sephadex LH-20 column eluting with MeOH afforded **58**, dicentrinone, (6.5 mg, 0.065 %). (Refer Scheme 3.10)

Physical data:

$C_{19}H_{13}NO_5$, yellow fine needles. Yield: 0.065 %.

Spectral data were identical to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis*; methanol extract of leaves of *Actinodaphne sesquipedalis* and hexane extract of fruits of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.1.** Please refer (page 72).

Stigmasterol (60)

Fractions 21-25 of first column chromatography (Sephadex LH-20 eluated with MeOH) were combined and rechromatographed using Sephadex LH-20 small gravity column (MeOH : chloroform, ratio 90 : 10) to give another 10 fractions. Fractions 7-10 obtained were evaporated to dryness and further purified via preparative TLC repeatedly three times with the solvent system DCM : MeOH (99 : 1, 97 : 3, 95 : 5). Then, the white solid yield was recrystallise with MeOH to give needle crystals **60** as stigmasterol (10.0 mg, 0.1 %). (Refer Scheme 3.10)

Physical data:

$C_{29}H_{48}O$, white powder, $[\alpha]^{25}_D$ = -20.6° (c= 0.15, $CHCl_3$). Yield: 0.1 %

Spectral data were identical to stigmasterol (**60**) isolated from hexane extract of bark of *Phoebe grandis* and hexane extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **3.7.1.2**. Please refer (page 73-74).

3.8 Bioassay and Chemical Assay Procedures.

Crude extracts and selected pure compounds of *Phoebe grandis, Phoebe tavoyana* and *Actinodaphne sesquipedalis* were screened for biological activities. The biological tests conducted were cytotoxicity, antioxidant, antibacterial and antiplasmodial activities.

3.8.1 Cytotoxic Assay

Colorimetric assay is a standard assay in cytotoxic screening to monitor the living cells. A rapid colorimetric assay, based on the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was first developed in 1983 by Mosman (Mosmann, 1983).

The crude extracts and isolated compounds from *P. grandis, P. tavoyana* and *A. sesquipedalis* were screened for their cytotoxic activity by using Microculture Tetrazolium Salt (MTT) assay (Mosmann, 1983). The concentration for crude extract was 10 mg/mL and 1 mg/mL for pure compounds.

3.8.1.1 Cell lines

The cell lines used were human hepatocarcinoma (HepG2), human estrogen receptor (ER+) positive breast cancer (MCF7) and human ovarian cancer cell line (Caov-3). The cell lines were obtained from American Type Culture Collection ATCC (Rockville, MD). The cells were stored in liquid nitrogen tank at the Cell Culture Laboratory, Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala Lumpur.

3.8.1.2 Reviving cells

Cells in cryovials from the liquid nitrogen tank were thawed in between palms of hands or warmed rapidly by placing it into a 37°C water bath. The cryovials were sprayed with 70 % EtOH before opening the laminar flow cupboard. Once thawed, the cells were transferred into a sterile centrifuge tube, then add in 10 mL of medium/serum

(10% of serum in 90% of medium) and spun down at 1000 rpm (200 x g) for 10 minutes to draw off DMSO, then, the supernatant was removed. The pellet of cells was immediately transferred into T-flask (cultured flask) containing 10 mL medium Roswell Park Memorial Institute media, RPMI-1640 (Sigma, USA) containing 10 % fetal calf serum (Flow Lab., Australia), 100 IU/mL penicillin (Flow Lab., Australia) and 100 g/mL streptomycin (Flow Lab., Australia) as a complete growth media (CGM). The pellet was gently resuspended in the medium to avoid clumping of cells. The cells were cultured in an incubator at 37°C with 5 % CO_2 in humidified atmosphere. The culture was subculture 1:1 every 24 hours to remove debris, until a clean culture was obtained. Subculturing was done by removing 5 mL of the culture and adding another 5 mL of fresh CGM.

3.8.1.3 Maintenance of Cell Culture

Human estrogen receptor (ER+) positive breast cancer (MCF7), human ovarian cancer cell line (Caov-3) and human hepatocarcinoma (HepG2) were maintained in RPMI 1640 (Sigma, USA) medium supplemented with 10 % of fetal calf serum (FCS), 100 IU/mL of penicillin and 100 g/mL streptomycin (Flow Lab., Australia) as a complete growth media (CGM). Cells were cultured as monolayers and maintained in 25 cm^3 flask with 10 mL of CGM in incubator at 37°C with 5 % CO_2 in humidified atmosphere throughout the study. The cells were subcultured when reached up to 80-90% confluency.

Anchorage-dependent cells such as MCF7, Caov-3 and HepG2, need trypsinisation to detach the cells from the surface of culture flask. The old medium from cultured cells was removed and followed by adding 2-3 mL phosphate buffer saline (in 25 mL culture

flask) to remove traces of serum which may inhibit the action of trypsin and discard the solution. Then, 2-3 mL (for 25 mL of culture flask) 0.25% (v/v) of trypsin (Accutase, PAA, Austria) was added and incubated at 37°C for about 5-10 minutes (until most of the cells detach). A gentle shaking was used to help the cell monolayer detach from the flask surface. CGM was added to the flask containing detached cells to stop the trypsin reaction. Cells were resuspended gently and subcultured. Cultures were maintained in RPMI 1640 with 10% fetal calf serum, at 37˚C with CO_2 in humidified atmosphere.

3.8.1.4 Cytotoxicity analysis of crude extracts and compounds by the MTT assay

Twenty crude extracts and compounds were tested for cytotoxic activity by using Microculture Tetrazolium Salt (MTT) assay (Mosmann, 1983). The stock solution of crude extracts and compounds were prepared at concentration of 10 and 1 mg/mL, respectively in dimethylsulfoxide (DMSO). Cytotoxic assays were performed in 96-flat bottom microwell plates. Each well was filled with 100 µL of varying concentrations of sample solution (100, 50, 25, 12.5, 6.25, 3.135, 1.5625) µg/mL were prepared from substock solutions by serial dilution in RPMI-1640 in each microtitre plate well. Each well was then added with 100 µL of exponentially growing cell suspension in a complete growth medium at the concentration of 5 x 10^5 cells/mL. Untreated cells were used as control and included for each sample. The assays were performed in triplicate and the culture plates were incubated for 24 hours at 37˚C in 5% CO_2 humidified incubator. After incubation, the fractions of surviving cells were determined relative to the untreated cell population by the colorimetric MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenytetrazolium bromide) assay. In this method, the viability of cells were determined by measuring the amount of blue formazan crystals formed after the freshly prepared 20 µL of MTT solution (5 mg in 1 mL PBS, phosphate buffer saline) was

added to each well followed by 4 hours incubation at 37˚C. Then, 170 µL of the remaining supernatant was removed and 100 µL of DMSO was added to each well and mixed thoroughly to dissolve the formed blue crystal formazan. The plate was then left for 30 minutes of incubation to ensure all crystals were dissolved, before reading the absorbance (OD) of each well at 570 nm wavelength by using an ELISA microplate reader. Several positive controls such as tamoxifen, paclitaxel and doxorubicin were used in the experiment. The percentage of cell viability was determined as follows:

$$\% \text{ Viability} = \frac{OD_{sample}}{OD_{control}} \times 100\ \% \qquad \ldots. \text{ Equation 1}$$

A plot of the percentage of cell viability against concentration of the extract or compound gives a measure of cytotoxicity. The cytotoxic index used was IC_{50}, which is concentration that causes 50% inhibition of the cell compared with untreated control. All tests were run in triplicate and average.

3.8.2 Antioxidant activity asssay

3.8.2.1 DPPH free radical scavenging activity

The technique using 96 micro-well plates was used with slight modification and based on the work described by Lee *et al*., (1998). Stock solutions of the crude extracts were prepared at 10 mg/mL in DMSO. The stock solution 10 mg/mL of the crude extract was then diluted into 1 mg/mL as a substock. The positive control used in this assay was ascorbic acid (Sigma, USA). Each well was filled in with 100 µL of diluent (DMSO). The first row of the 96 wells was filled with 100 µL of diluted sample (1 mg/mL). Concentration in the first row was 500 µg/mL of sample in each well and was serially

diluted (two fold dilution) in 96 micro well plates to give 6 series of concentrations, 500 µg/mL the highest to the lowest 15.63 µg/mL. Then, 10 µL of the DPPH (diphenyl-*p*-picrylhydrazine) solution (2.5 mg/mL in DMSO) was added to each well in. The DPPH solution should be kept in the dark at 4 °C. This procedure should not be done under direct light. The plate was shaken gently to ensure thorough mixing before placed in a dark area. After 20 minutes, the optical density of the well was read using ELISA microplate Reader at wavelength 517 nm. Radical Scavenging Activity (% inhibition) was calculated using the following equation:

$$\text{Radical Scavenging Activity (\%)} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100\% \quad \text{Equation 2}$$

The IC_{50} value was determined as the concentration of each sample was required to give 50 % reduction of the DPPH radicals. All tests and analyses were run in triplicate and averaged. Colour of DPPH solution changed from purple to pale yellow colour indicates the activity of antioxidant properties of the samples. Lower optical density of spectrophotometer of the reaction mixture indicated higher free radical scavenging activity.

3.8.2.2 FRAP assay (ferric reducing ability of plasma)

(a) Reagent for the FRAP assay

i. Acetate buffer 300mM, pH 3.6

Sodium acetate trihydrate (3.1 g) was added to 16 ml of glacial acetic acid and the solution were diluted with distilled water till the volume become 1 L.

ii. A solution of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) was prepared in 40 mM HCl.

iii. 20mM $FeCl_3.6H_2O$ (M.W: 270.30)

Iron chloride, $FeCl_3$ (0.027 g) was added to 5 ml of distilled water.

The working FRAP reagent was prepared by mixing (i), (ii) and (iii) in the ratio of 10:1:1 (v/v/v) just before testing.

(b) Standard was $FeSO_4.7H_2O$, 0.1-1.5 mM in methanol.

Iron sulphate (0.027 g) was added to 10 ml distilled water.

All solutions were used on the day of preparation. All the reagents were purchased from Merck (Germany) company.

(c) Procedure

The ability to reduce ferric ions Fe^{3+} to its ferrous form, Fe^{2+} at low pH was measured using a modified version of the method described by Benzie & Strain, (1996). An aliquot (10 µl) of extract (with appropriate dilution) was added to 300 ul of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 . 6H_2O$ solution) and the reaction mixture was incubated in a water bath at 37^oC . The increase in absorbance at 593 nm was measured immediately (t = 0 minute) and the second reading was measured after 4 minutes. The antioxidant capacity based on the ability to reduce ferric ions (Fe^{3+}) of the extracts

sample to ferrous ion (Fe^{2+}) was expressed as form an intense blue Fe^{2+}-TPTZ complex. Increased absorbance of the reaction mixture indicates the increase in reducing power.

3.8.3 Antibacterial activity asssay

Antibacterial activity of the plant extracts and the isolated compounds were tested by a modified disc diffusion method Bauer *et al.,* (1966).

3.8.3.1 Microorganisms

The following bacterial strains were employed in the screening of the extracts:
Bacillus subtilis (B145-IMR culture), *Staphylococcus aureus* (S1434-IMR culture), *Staphylococcus epidermidis* (a clinically isolated strain-UMMC), *Escherichia coli* (ATCC 25922), *Salmonella typhi* (clinically isolated strain-UMMC), and methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1720).

The *in vitro* antibacterial activity of the alkaloid compounds was evaluated against five pathogenic microorganisms, including three Gram positive bacteria: *Staphylococcus epidermidis* (a clinically isolated strain-UMMC), *Staphylococcus aureus* (S1434-IMR cultured), *Bacillus subtilis* (B145-IMR culture) and two Gram negative bacteria: *Pasteurella multocida* (a clinically isolated strain-UMMC) and *Enterobacter cloacae* (a clinically isolated strain-UMMC). All the strains were stored in the appropriate medium before use.

3.8.3.2 Disc diffusion assay

(a) Disc diffusion assay of crude extracts and compounds

Antibacterial activity of the crude extract from *Phoebe* and *Actinodaphne* species and isolated compounds from *Phoebe grandis* leaves was determined by disc diffusion method (Bauer *et al*., 1966; Hashim *et al.*, 2012) with slight modification in terms of sample concentration, volume of sample loaded and use of paper discs. The bacteria were cultured at 37 °C for overnight in nutrient broth and potato dextrose broth, respectively. The concentrations of the cultures were adjusted turbidometrically at a wavelength of 600 nm which give 10^5-10^6 colony forming units (CFU) per ml.

The medium agar (Nutrient Agar for bacteria) was prepared in distilled water according to manufacturer instructions. Nutrient broth was prepared by mixing 5.0 g NaCl, 3.0 g wheat extract, 5.0 g peptone, 15 g agar and 50 ml distilled water by stirring. This mixture was then transferred into four universal bottles and autoclaved for 15 minutes at 15 psi. The autoclave was allowed to cool (2 hours) and the medium was removed while still hot. By using a wire loop, a loopful of each microbe was taken from a slope culture (bacteria stock) and inoculated to the broth.

The agar medium was prepared by mixing 1.6 g broth, 3.0 g of nutrient agar with 200 ml of distilled water. The plates were prepared by adding 0.1 ml the bacteria grown in nutrient broth culture to the agar medium and this liquid medium was then poured into the sterile petri dishes and shake slowly for better mixing (15 ml/plate). The agar was allowed to set in at 37°C.

The crude extracts and isolated compounds to be tested were dissolved in dimethylsulphoxide (DMSO) at concentration of 100 and 1 mg/mL, respectively. For the purpose of screening, 10 µl of each sample solution was loaded on Whatman No. 1 filter paper disc (Ø 6mm) and dried under sterile conditions to remove the solvent of stock solution. The dried disc were then placed on the (nutrient or potato dextrose agar) previously inoculated agar surface. The agar plates were then inverted and incubated for 24 hours at 37°C. Antibiotics streptomycin sulphate and gentamycin (10 µg/disc) were used as positive control and DMSO as negative control in this assay. The antimicrobial activity was recorded by measuring the zone of inhibition (IZ) in mm around each of the disc, against the test organisms. The strength of activity was categorised as strong if the inhibition zone diameter is equal or more than 15 mm, moderate activity for diameter ranging from 10.0 to 14.5 mm and weak activity for diameter equal or less than 9.5 mm. An average zone of inhibition was calculated for the three replicates. An inhibition zone of 8 mm or greater was considered as good antibacterial activity (Ali *et al.*, 2001).

3.8.4 Antiplasmodial Test Against *Plasmodium falcifarum* Strains

This procedure for assessing compound efficacy against *Plasmodium falcifarum in vitro* uses chloroquine strain as a marker for inhibition of parasite growth, was continuously cultured according to the methods describe by Trager & Jensen, (1976). Many alternative protocols exist, including ones based on microscopic detection of Giemsa-stained slides, and parasite lactate dehydrogenase (pLDH) activity was used to measure parasite viability, by using flow cytometry (Makler *et al.*, 1993). The *in vitro* assays were performed as described by Budimulja *et al*. (1997).

3.8.4.1 Preparation of the Antiplasmodial Test

(a) Parasite Strain

The recommended strains to test antiplasmodial activity against drug-sensitive line such as 3D7 (West Africa), D6 (Sierra Leone) or D10 (Papua New Guinea), as well as drug-resistant line such as W2 or Dd2 (both from Indochina), FCB (SE Asia), 7G8 (Brazil) or K1 (Thailand).

(b) Malaria Culture Media

RPMI 1640 medium containing L-glutamine (Catalog number 31800, Invitrogen), 50 mg/L hypoxanthine, 25 mM HEPES, 10 µg/mL gentamicin, 0.225 % $NaHCO_3$ and either 10 % human serum or 0.5 % Albumax I or II (purified lipid-rich bovine serum albumin, Invitrogen). Medium is typically adjusted to a pH of 7.3 to 7.4.

3.8.4.2 Determination of the antimalarial activity

The antimalarial activity of the isolated compounds was determined by the procedure describe by Budimulja *et al*., (1997). In brief, stock solutions of the samples were prepared in DMSO (final DMSO concentrations of < 0.5 % and kept at -20°C until used) and were diluted to the required concentration with complete medium (RPMI 1640 supplemented with10 % human plasma, 25 mM HEPES and 25 mM $NaHCO_3$) until the final concentrations of samples in culture plate wells were 10; 1; 0.1; 0.01; 0.001 µg/mL. The malarial parasite *P. falciparum* 3D7 clone was propagated in a 24-well culture plates. Growth of the parasite was monitored by making a blood smear fixed with MeOH and stained with Giemsa stain. The antimalarial activity of each

compound was expressed as an IC_{50} value, defined as the concentration of the compound causing 50 % inhibition of parasite growth relative to an untreated control. The percentage of growth inhibition was expressed according to following equation:

Growth inhibition % = 100 – [(test parasitemia / control parasitemia) x 100].

… Equation 3

Chloroquine: IC_{50} 0.0069 µg/mL.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical constituents

In this study, twenty extracts of two *Phoebe* species namely, *Phoebe grandis* and *Phoebe tavoyana*; and one from *Actinodaphne sesquipedalis* were preliminary screened for their cytotoxicity, radical scavenging activities, antibacterial, and antiplasmodial. Based on bioactivity results from the crude extracts and previous phytochemical studies family Lauraceae contain lots of alkaloids that generally known to be biologically active and many natural drugs are alkaloids. The extracts were chosen for detail investigation or further analysis which involves chromatographic work, isolation of chemical compounds, structural elucidation using spectroscopic methods and further bioassay screenings for some of the identified compounds.

The chemical constituents of three species; *Phoebe grandis*, *Phoebe tavoyana* and *Actinodaphne sesquipedalis* were investigated in detail. The plant extracts were separated by various chromatographic techniques and the isolated pure compounds were submitted for spectra analysis including UV, IR, MS and NMR. The data were analysed, interpreted and compared with literature values. Table 4.1 showed the various compounds isolated and structurally identified from the plant study.

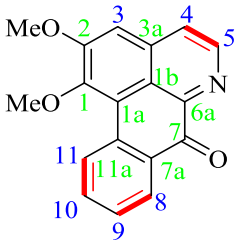
Table 4.1: Compounds isolated from *Phoebe* and *Actinodaphne* species.

Source	Part	Isolated Compounds	Yield (mg)	Yield (%)
KL 5440				
Phoebe grandis	leaves	Lysicamine (**54**)	12.5	0.16
Dichloromethane extract		Litsericinone (**55**)	9.0	0.11
(acid-base)		8,9,11,12-tetrahydromecambrine (**56**)	7.0	0.09
		Hexahydromecambrine A (**57**)	6.5	0.08
		Dicentrinone (**58**)	6.0	0.08
KL 4994				
Phoebe grandis	bark	β-sitosterol (**59**)	7.0	0.53
Hexane extract		Stigmasterol (**60**)	6.2	0.47
Dichloromethane extract	bark	Boldine (**5**)	5.5	0.14
(acid-base)		N-methyllaurotetanine (**51**)	8.0	0.20
		Reticuline (**61**)	10.0	0.25
		Laurolitsine @ Norboldine (**6**)	7.0	0.18
KL 5225				
Phoebe tavoyana,	leaves	Laetanine (**62**)	8.0	0.10
Dichloromethane extract		Roemerine (**20**)	5.0	0.06
(acid-base)		Laurolitsine (**6**)	6.5	0.08
		Tavoyanine A (**63**)	8.0	0.10
		Tavoyanine B (**64**)	7.0	0.09
		Boldine (**5**)	5.5	0.07
		Sebiferine (**22**)	5.0	0.06
HIR 011				
Actinodaphne sesquipedalis	leaves	β-sitosterol (**59**)	5.5	0.06
		Dicentrine (**52**)	4.0	0.04
Hexane extract		N-methyllaurotetanine (**51**)	6.0	0.07
		Stigmasterol (**60**)	5.0	0.06
MeOH extract	leaves	β-sitosterol (**59**)	7.0	0.07
		Dicentrine (**52**)	10.0	0.01
		Dicentrinone (**58**)	5.5	0.06
		Boldine (**5**)	5.0	0.05
		Norisocorydine (**65**)	7.5	0.08
		Laurolitsine @ Norboldine (**6**)	6.5	0.07
HIR 011				
Actinodaphne sesquipedalis	fruits	Dicentrine (**52**)	62.0	8.86
		Liriodenine (**19**)	3.0	0.04
Hexane extract		Dicentrinone (**58**)	6.3	0.09
		β-sitosterol (**59**)	5.0	0.07
MeOH extract	fruits	Dicentrine (**52**)	8.0	0.08
		β-sitosterol (**59**)	10.0	0.10
		Dicentrinone (**58**)	6.5	0.07
		Stigmasterol (**60**)	10.0	0.10

4.2 Alkaloids isolated from the leaves of *Phoebe grandis* (KL 5440)

The present study reports the isolation of two new naturally occurring compounds of proaporphine alkaloids, litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**); one known proaporphine alkaloid, hexahydromecambrine A (**57**); and two oxoaporphine alkaloids, lysicamine (**54**) and dicentrinone (**58**). All isolated compounds are reported from the leaves of *Phoebe grandis* (KL 5440) for the first time.

4.2.1 Lysicamine (54)



54

Lysicamine (**54**) was obtained as a yellow amorphous solid. $[\alpha]_D^{29} = -25^\circ$ (c = 0.5, $CHCl_3$). The mass spectrum (Figure 4.1) showed the $[M+H]^+$ peak at *m/z* = 292.0963, which corresponds to a molecular formula of $C_{18}H_{13}NO_3$. (calcd. for $C_{18}H_{14}NO_3$, 292.0929). The UV spectrum showed absorption maxima at 236, 267, 360 and 396 nm, indicating the existence of a highly unsaturated oxoaporphine chromophore (Chen-Loung *et al.*, 1976). The IR spectrum (Figure 4.2) showed a conjugated carbonyl absorption at 1659 cm^{-1}.

The ^{1}H-NMR spectrum (Figure 4.3) showed two distinct methoxyl peaks at δ 4.00 and δ 4.08, which were probably situated at C-1 and C-2. H-3 appeared as a singlet at δ 7.21. Two doublets (*J* = 5.2 Hz) typical of the H-4 and H-5 signals of an oxoaporphine were observed at δ 7.78 and δ 8.88, respectively. The H-5 proton was deshielded by the

neighbouring N atom. The methoxyl of C-1 was shielded compared to that of C-2 since it experiences an anisotropic effect from the opposite ring D. A very downfield signal at δ 9.16 appeared as doublet of doublet with J_1 = 8.4 and J_2 = 1.4 belongs to H-11 which was due to extensive hydrogen bonding with the methoxyl group attached to C-1 and also the deshielding effect of the facing aromatic ring A. In addition, a doublet of doublet was observed at δ 8.57 (1H, *dd*, J_1 = 8.4 Hz, J_2 = 1.4 Hz; H-8) which experienced a deshielding effect from the neighbouring C-7 carbonyl group. The peak appeared as doublet of triplet at δ 7.56 with J_1 = 8.5 Hz and J_2 = 1.6 Hz was assigned for H-9 whereas H-10 resonated at δ 7.75 as doublet-triplet with J_1 = 8.5 Hz and J_2 = 1.6 Hz.

The ^{13}C-NMR spectrum (Figure 4.4) gave a total of eighteen carbons which validated the molecular formula of $C_{18}H_{13}NO_3$. Analysis of the ^{13}C-NMR spectrum and DEPT spectrum (Figure 4.4) gave a total of nine aromatic quaternary carbons, seven aromatic methines, two methoxyls, a carbonyl group at C7 and no methylene signals appeared.

The COSY spectrum (Figure 4.5) showed correlations between H4/H5, H8/H9 and H10/H11. In the HMBC spectrum (Figure 4.7) revealed H_{11} showed long range heteronuclear interactions with the C1a, C9, C11a and C7. The presence of the carbonyl group at C7 was confirmed based on the HMBC correlations of H11 and H8 to C7. The H4 showed long range correlations to the signals at C1b, C3 and C5. Other correlations of HMBC coupling pattern are summarised in the Table 4.2 and Figure 4.7. The structure also supported by HSQC (Figure 4.6) that showed ^1J heteronuclear single quantum correlation.

The 2D NMR data were not reported in the previous work (Chang *et al*., 2000; Zhang *et al.*, 2002). On the basis of the above evidence and comparison with the literature values, it is concluded that alkaloid **54** is an oxoaporphine alkaloid lysicamine (**54**) (Table 4.2). This oxoaporphine lysicamine (**54**) alkaloid is ubiquitous in the Anonaceae family, and now is also found in this Lauraceae family. Interestingly, this compound lysicamine (**54**) is reported here for the first time present in the leaves of *Phoebe grandis*.

Table 4.2: ^{1}H-NMR (400 MHz),^{13}C-NMR (100 MHz) and 2D (HMBC and HSQC) NMR data of lysicamine (**54**) and the literature data.

	54 in $CDCl_3$				* in $CDCl_3$	** in $CDCl_3$
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	151.0	-	-	-	152.2
1a	-	118.8	-	-	-	120.0
1b	-	121.2	-	-	-	122.3
2	-	155.8	-	-	-	157.0
3	7.21 (1H, *s*)	105.4	C_{1b},C_1,C_2	H3	7.24 (1H, *s*)	106.6
3a	-	134.5	-	-	-	135.7
4	7.78 (1H, *d*, *J*=5.2)	122.6	C_3,C_{1b},C_5	H4	7.82 (1H, *d*)	123.8
5	8.88 (1H, *d*, *J*=5.2)	144.0	C_4,C_{3a},C_5	H5	8.93 (1H, *d*)	145.2
6a	-	144.3	-	-	-	145. 4
7	-	181.7	-	-	-	182.9
7a	-	133.3	-	-	-	132.2
8	8.57 (1H, *dd*, J_1=8.4, $J_{2=}$1.4)	127.8	C_{7a},$C_{7,}$ $C_{11a,}C_{10}$	H8	8.59 (1H, *d*)	129.0
9	7.56 (1H, *dt*, J_1=8.52, $J_{2=}$1.64)	127.9	C_8,C_{11}, C_{11a}	H9	7.58 (1H, *dt*)	129.1
10	7.75 (1H, *dt*, J_1=8.52,$J_{2=}$1.64)	133.4	C_8,C_9, C_{11},C_{10}	H10	7.78 (1H, *dt*)	134.6
11	9.16 (1H, *dd*, J_1=8.4, $J_{2=}$1.4)	127.4	C_{1a},C_9, C_{11a},C_7	H11	9.19 (1H, *dd*)	128.6
11a	-	131.0	-	-	-	134.5
1-OCH_3	4.00 (3H, *s*)	59.7	C_1,C_2	$3H_{1\text{-OMe}}$	4.00 (3H, *s*)	60.8
2-OCH_3	4.08 (3H, *s*)	55.2	C_1,C_2	$3H_{2\text{-OMe}}$	4.11 (3H, *s*)	56.4

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.
* (Chang *et al.*, 2000).
**(Zhang *et al.*, 2002).

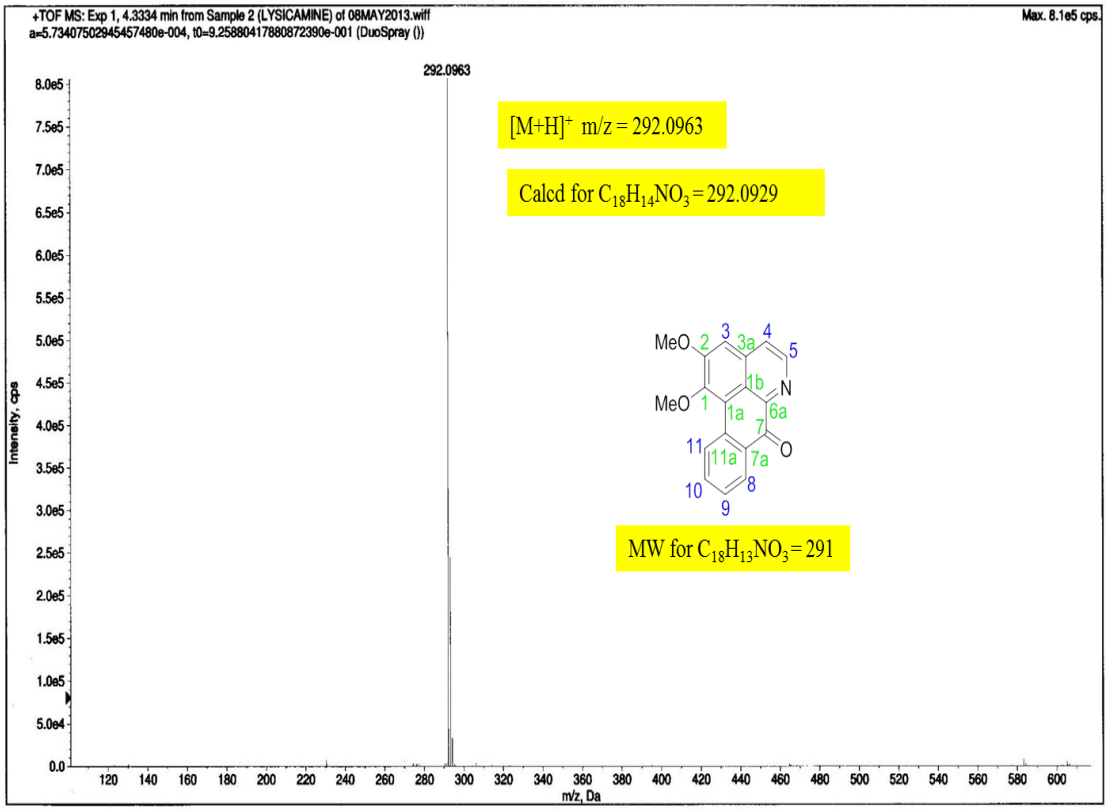


Figure 4.1: LCMS Q-TOF- MS spectrum of lysicamine (**54**)

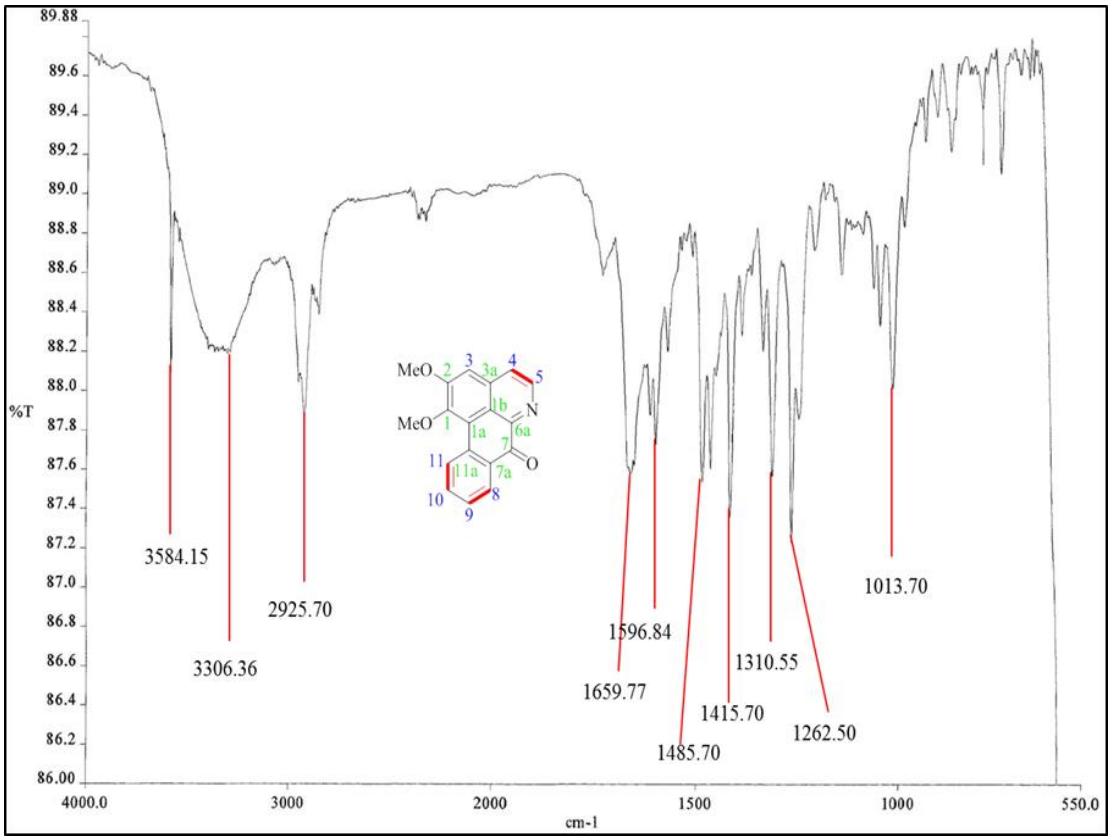


Figure 4.2: IR spectrum of lysicamine (**54**).

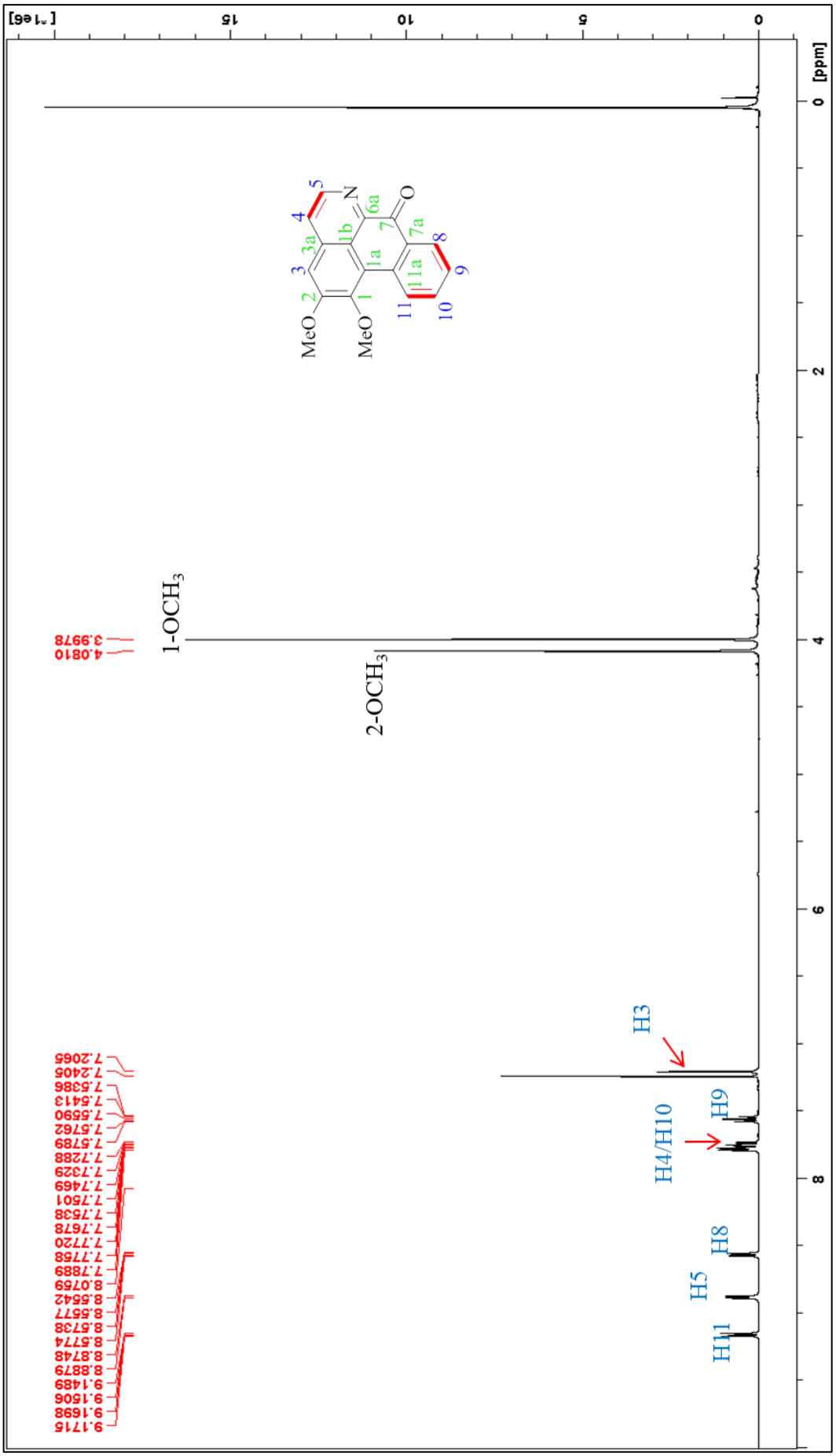


Figure 4.3: ^{1}H NMR spectrum of lysicamine (**54**)

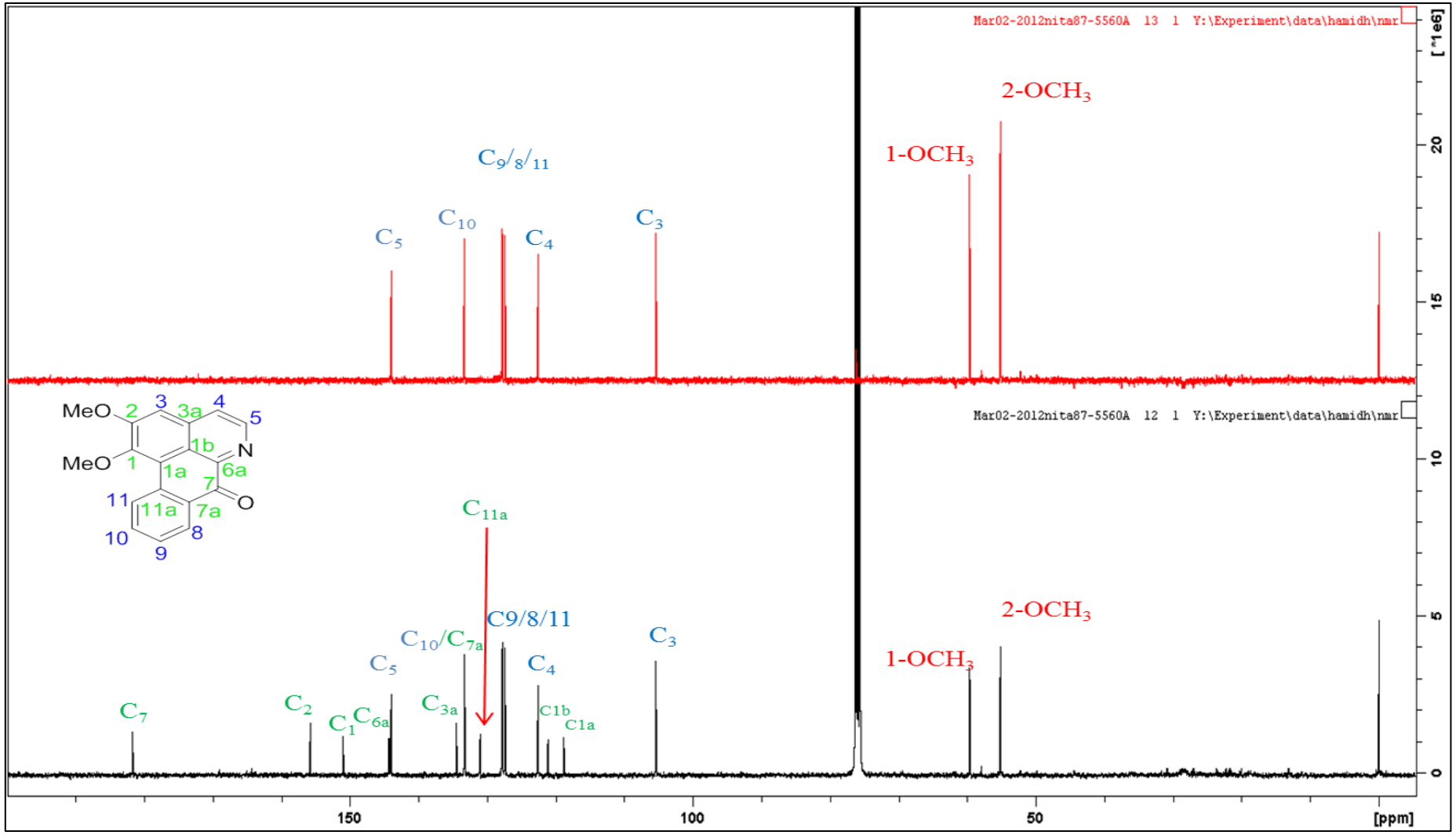


Figure 4.4: ^{13}C and DEPT 135 spectrum of lysicamine (**54**).

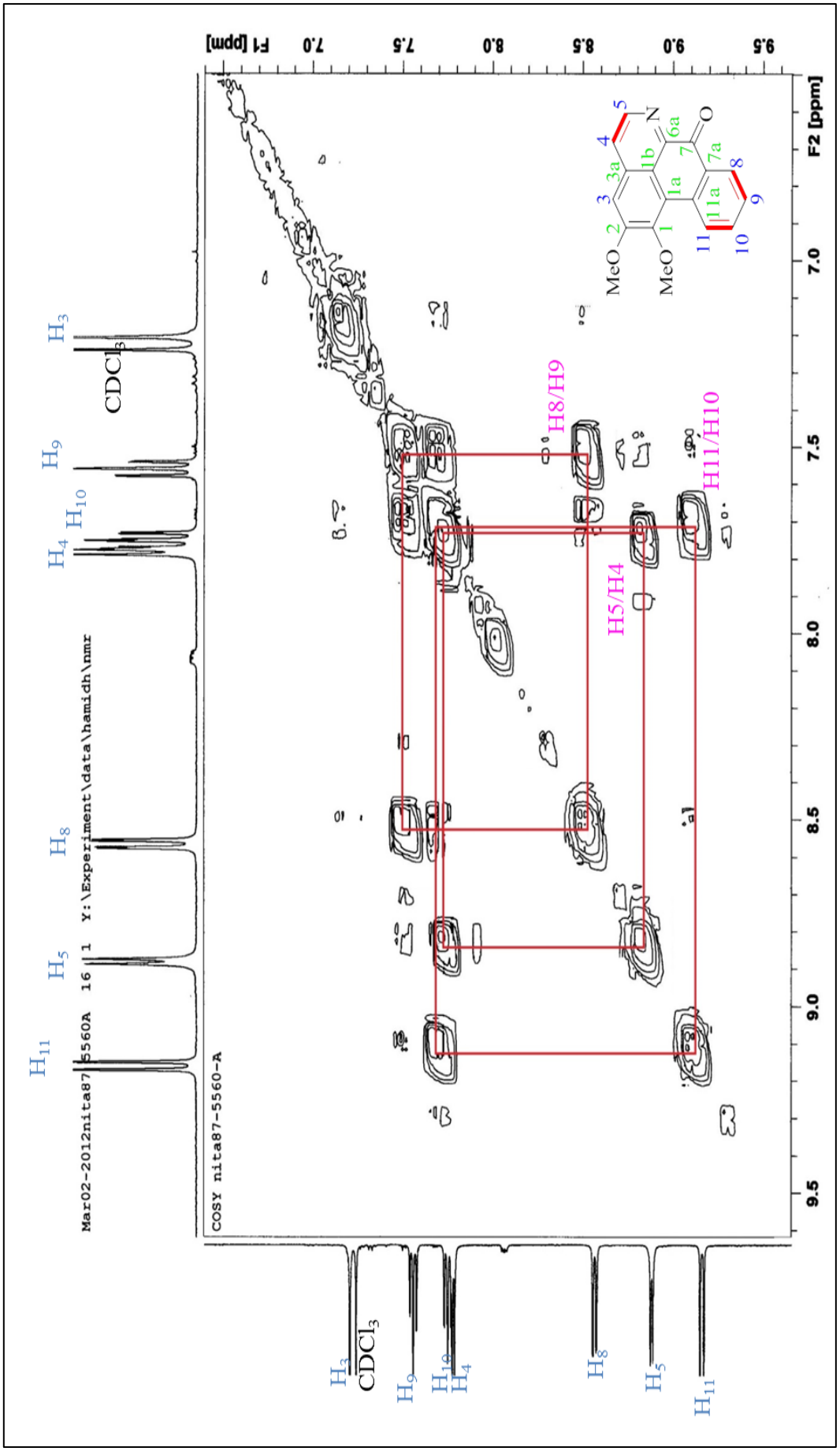


Figure 4.5: An expanded COSY spectrum of lysicamine (**54**) showing the correlations between H11/H10, H5/H4 and H8/H9

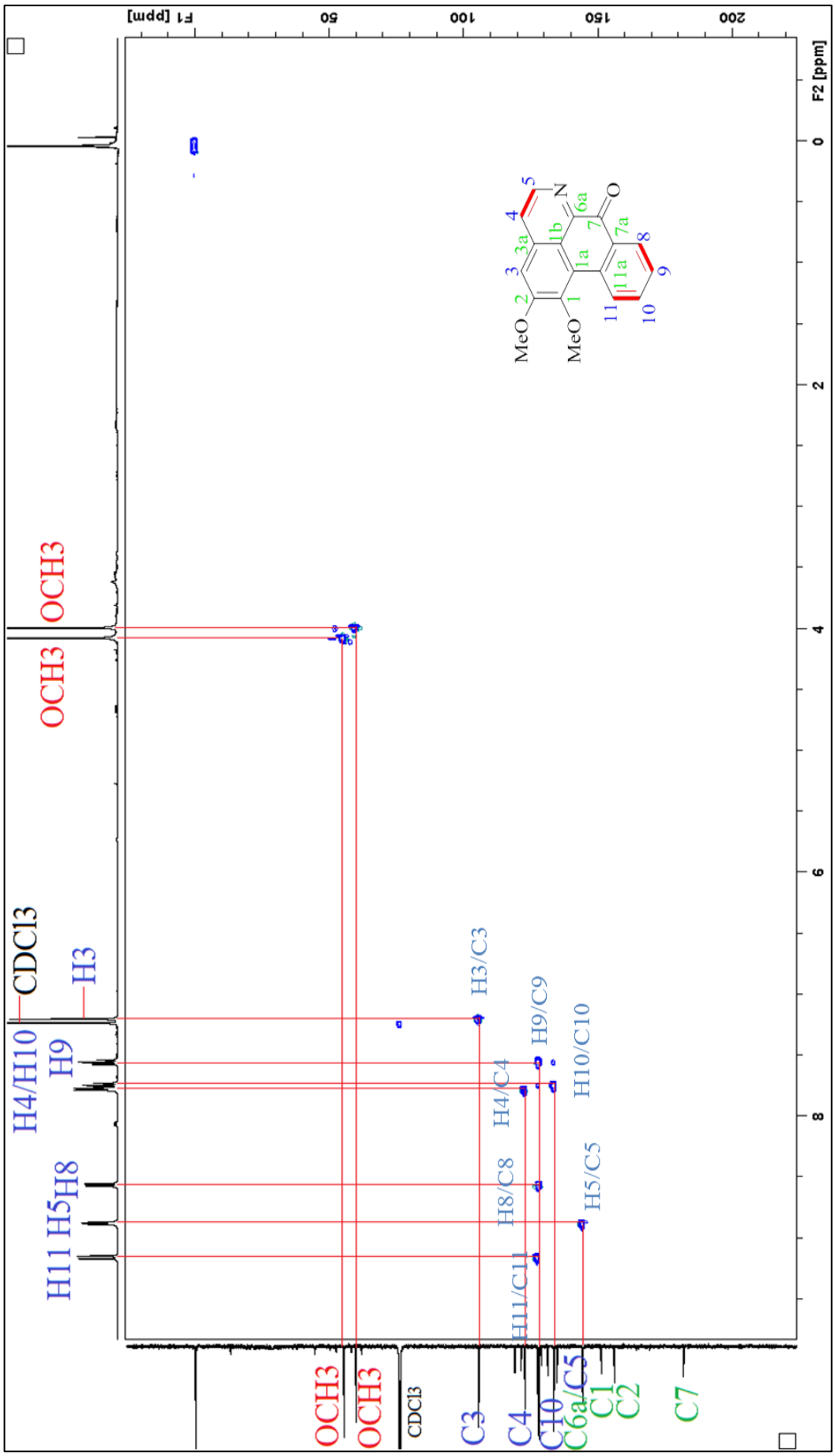


Figure 4.6: HSQC spectrum of lysicamine (**54**).

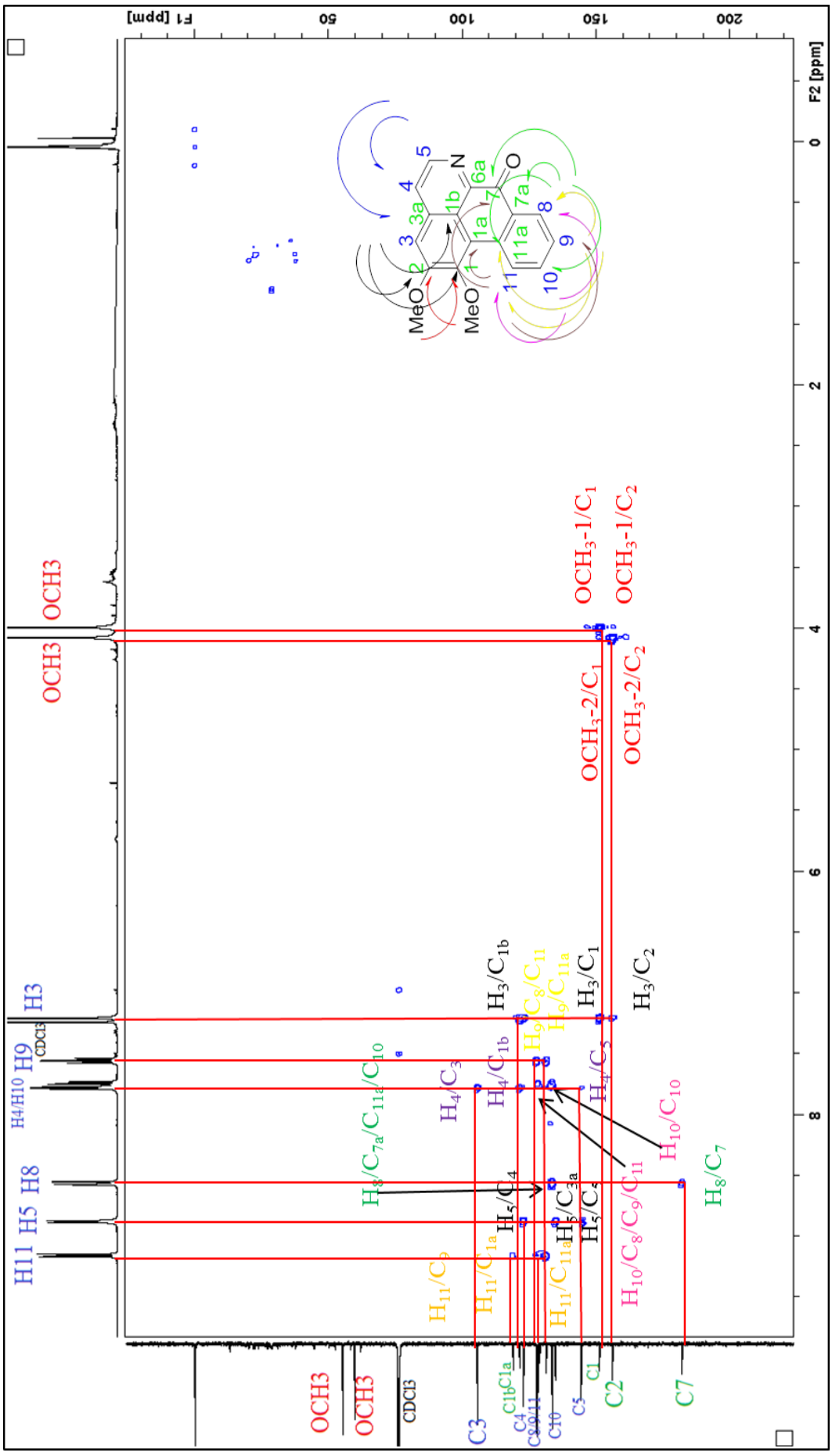
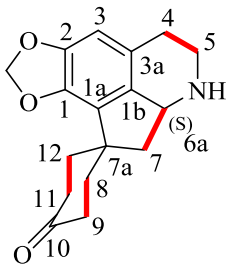


Figure 4.7: HMBC spectrum of lysicamine (**54**).

4.2.2 Litsericinone (55)



55

Litsericinone (**55**) was isolated as a yellow amorphous solid, $[\alpha]^{29}_{D} = -25°$ (c = 0.5, $CHCl_3$). This proaporphine alkaloid exhibited an $[M+H]^+$ peak in the LCMS-IT-TOF ESI (positive mode) mass spectrum (Figure 4.8) at *m/z* 286.1421 which correlated to the molecular formula of $C_{17}H_{19}NO_3$ (calcd. for $C_{17}H_{20}NO_3$, 286.1432). The UV spectrum revealed three peaks at 300, 236 and 207 nm, which indicated the existence of a conjugated system (Svatava *et al.,* 1975). The IR spectrum (Figure 4.9) revealed very significant carbonyl absorption at 1712 cm^{-1}. In addition, the presence of a methylenedioxyl group was proven by its characteristic absorption peaks at 1248 cm^{-1} and 934 cm^{-1}, which indicate asymmetric C-O-C stretching.

The ^{1}H-NMR spectrum (Figure 4.10) showed one aromatic proton singlet at δ 6.51 which may be ascribed to H-3. H-6a resonated at δ 4.26 (*dd, J, J'* = 9.8, 6.9Hz). The methylenedioxyl protons appeared as a pair of doublets at δ 5.92 and δ 5.88 with *J* = 1.2 Hz as geminal coupling constant of methylenedioxyl protons. Referring to the previous reported literature data (Charles *et al*., 1987; Mukhtar *et al*., 2008), *J* values for the methylenedioxyl protons of aporhine and proaporphine are in the range of 1.1 – 1.4 Hz. An integration of ^{1}H-NMR spectrum (Figure 4.11) showed the presence of 19 protons. The aliphatic protons of ring D resonated as multiplets between δ 1.91 to 2.68, while H-7 appeared as multiplets at δ 2.73 and δ 1.87 as showed in HSQC spectrum (Figure

4.14). The above observations were reinforced by COSY experiment (Figure 4.13) which showed correlations between vicinal protons; $H4_{ax}/H5_{ax}$, $H4_{eq}/H5_{eq}$, $H5_{ax}/H4_{ax}$, $H5_{eq}/H4_{eq}$, $H6a/H7_{eq}$, $H8_{eq}/H9_{eq}$, $H9_{eq}/H8_{eq}$, $H11_{ax}/H12_{ax}$ and $H12_{ax}/H11_{ax}$. Interestingly, H6a revealed correlation to H7eq suggesting the possibility that H6a is in an equatorial configuration.

The ^{13}C-NMR spectrum (Figure 4.12) of litsericinone (**55**) showed the presence of seventeen carbon atoms, whereas the DEPT experiment (Figure 4.12) displayed the presence of seven methylenes, a methylenedioxyl and two methine signals. The DEPT 135 and ^{13} C- NMR spectrum (Figure 4.12) revealed the C-10 carbonyl peak at δ 211.1 and the quarternary carbon showed five signal peaks between δ 123.9 to 148.9. The characteristic proaporphine quarternary C-7a spirocarbon appeared at δ 46.2.

The complete assignments of all protons and carbons (Table 4.3) were aided by the HSQC (Figure 4.14) and HMBC experiments (Figure 4.15). The proximity of H6a and $H7_{eq}$ was confirmed by the HMBC correlation peaks between $H5_{eq}$/C6a and $H7_{eq}$/C6a.

Therefore, based on the data and some related literature values, the structure of litsericinone (**55**) was established. This compound was isolated for the first time as new naturally occurring compound from plants and was published recently (Omar *et al.*, 2013) with a chiral center at position 6a as *S*-configuration.

Table 4.3: ^{1}H-NMR (400MHz), ^{13}C-NMR (100 MHz) and HMBC spectral data of litsericinone (**55**) in $CDCl_3$ (δ in ppm, J in Hz).

Position	^{1}H	^{13}C	HMBC (^{2}J,^{3}J)
1	-	141.1	
1a	-	132.4	
1b	-	123.9	
2	-	148.9	
3	6.51 *s*	106.9	C1, C1a, C2, C4
3a	-	126.7	
4	2.87 *m* (ax)	25.1	C3, C1b, C1a
	2.75 *m* (eq)		C3, C1b, C1a
5	3.55 *m* (ax)	43.9	C4, C6a, C1b
	3.16 *m* (eq)		C4, C6a
6a	4.26 *dd*	56.8	C5, C7
	(J = 6.9 Hz, J=9.8 Hz)		
7	2.73 *m* (ax)	44.1	C3, C3a, C1a
	1.87 *m* (eq)		C12, C7a, C6a
7a	-	46.2	-
8	2.68 *m* (ax)	38.5	C10
	2.41 *m* (eq)		C9, C7a, C10
9	2.50 *m* (ax)	36.2	C8, C7a, C10
	1.91 *m* (eq)		C11, C7a, C10
10	-	211.1	-
11	2.46 *m* (ax)	38.9	C10
	2.45 *m* (eq)		C12, C7a, C10
12	2.10 *m* (ax)	34.3	C9, C7, C7a, C3a, C10
	2.00 *m* (eq)		C9, C7a, C3a, C10
Methylenedioxy	5.92 *d* (J=1.2 Hz)	100.9	C1,C2
(O-CH_2-O)	5.88 *d* (J=1.2 Hz)		C1,C2

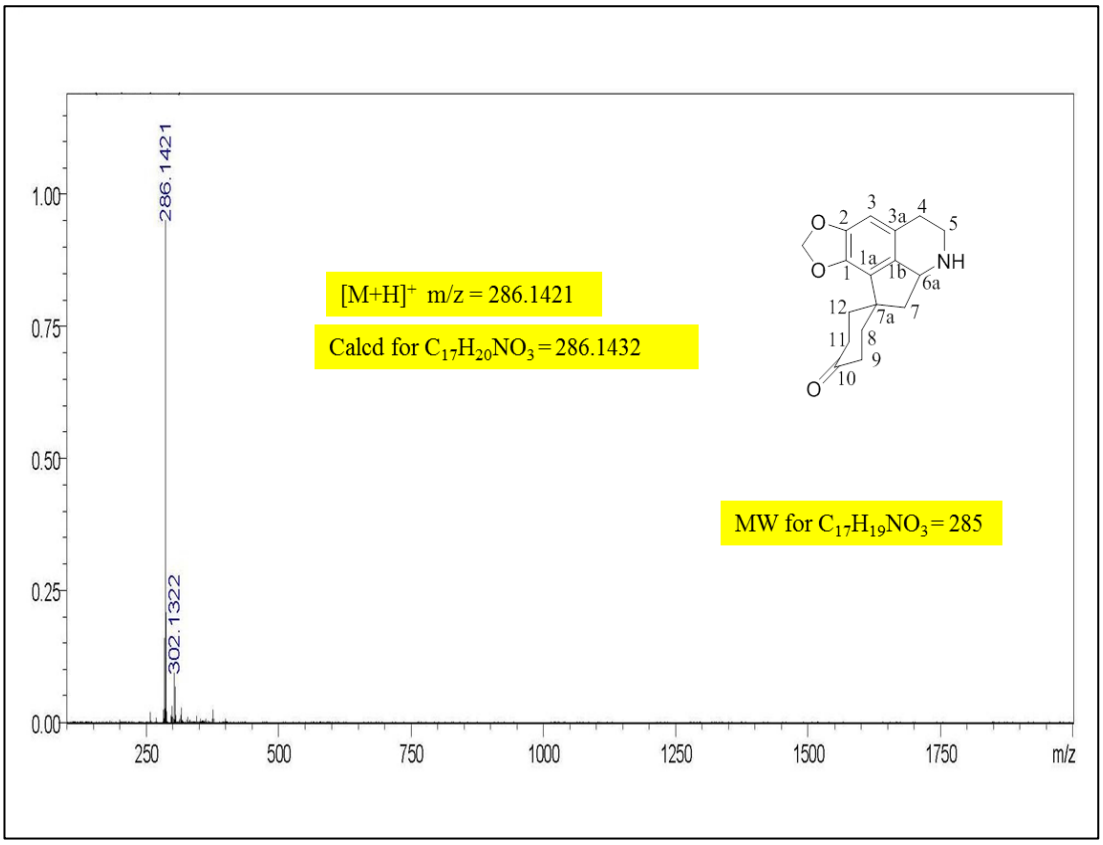


Figure 4.8: LCMS IT TOF- MS spectrum of litsericinone (**55**)

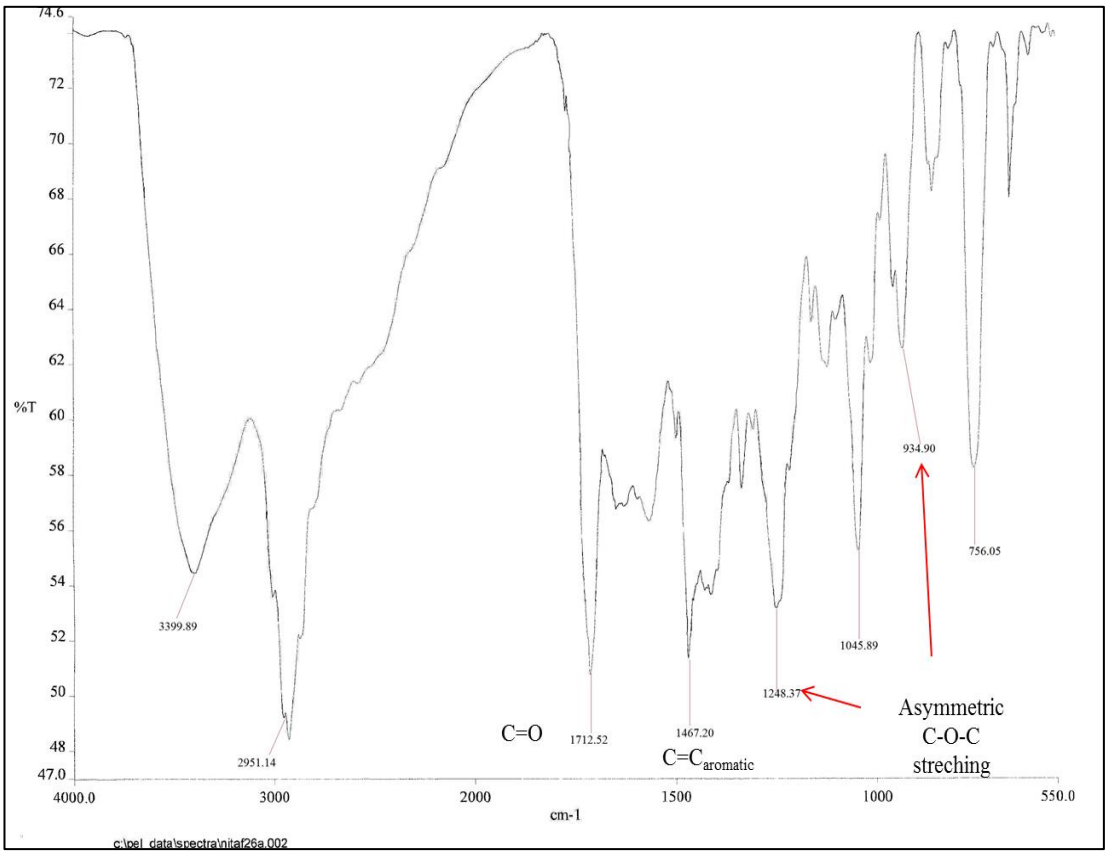


Figure 4.9: IR spectrum of litsericinone (**55**).

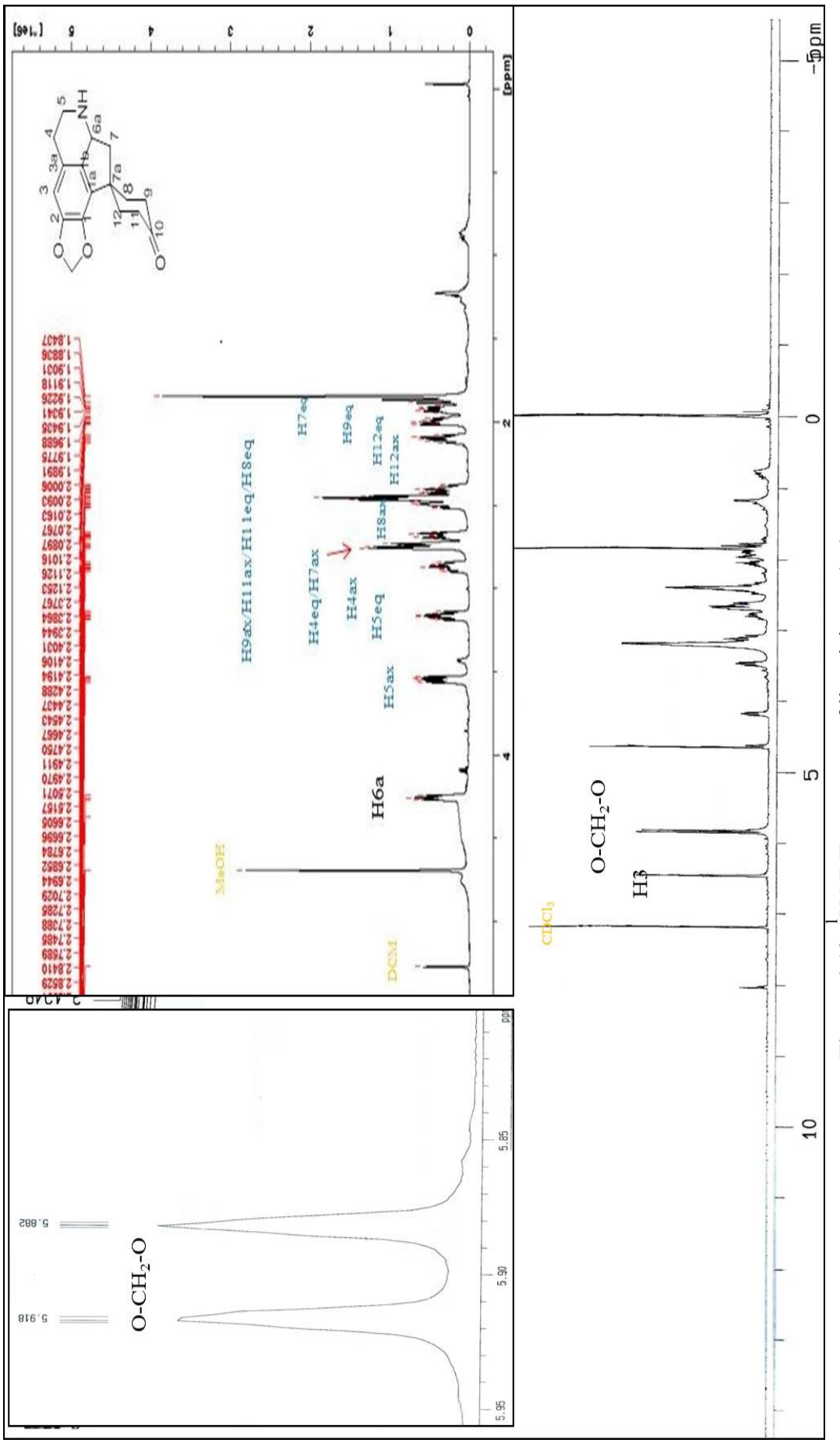


Figure 4.10: ^{1}H NMR spectrum of litsericinone (**55**)

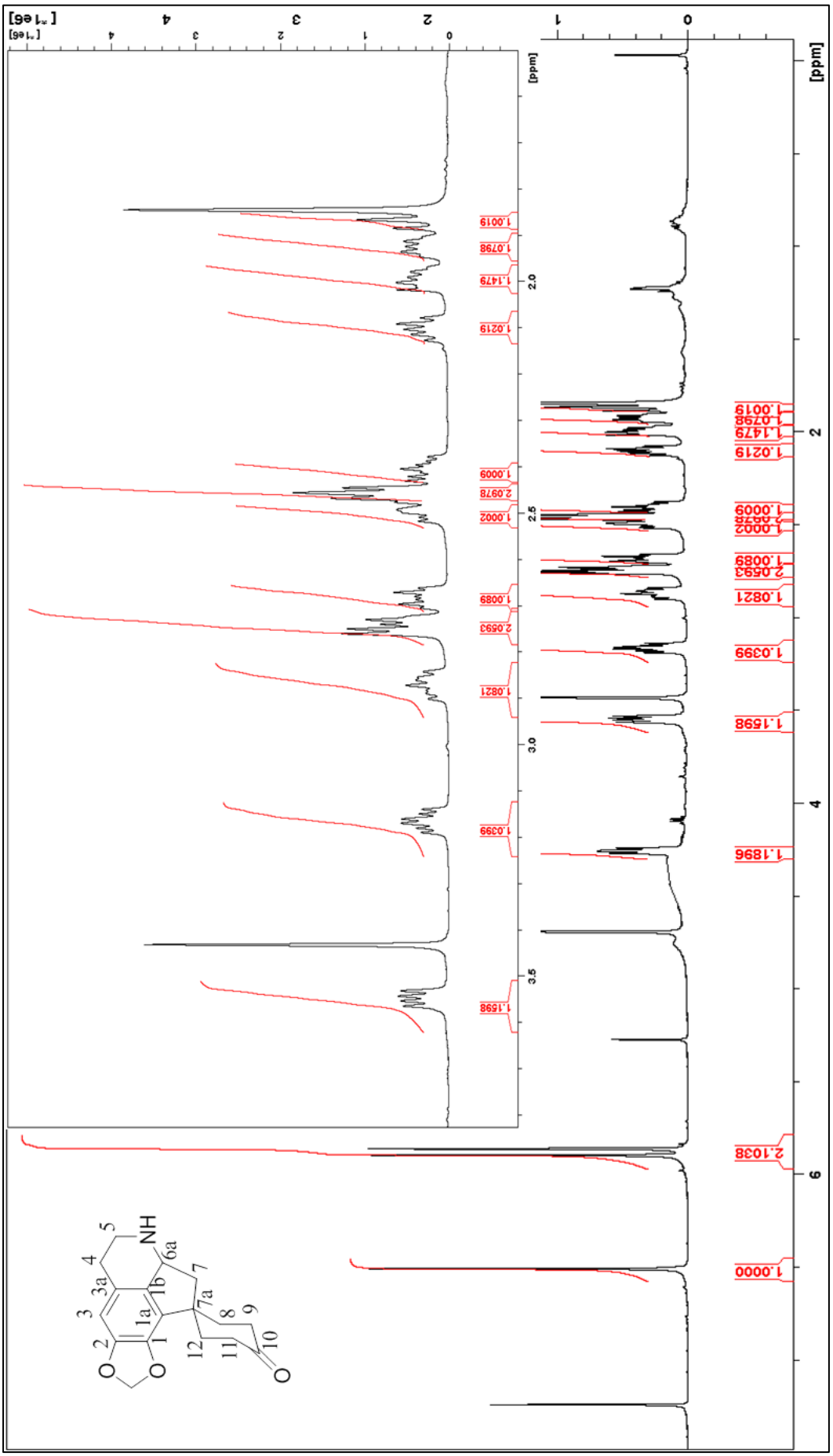


Figure 4.11: An integration of ^{1}H NMR spectrum of litsericinone (**55**), gives the total of 19 protons.

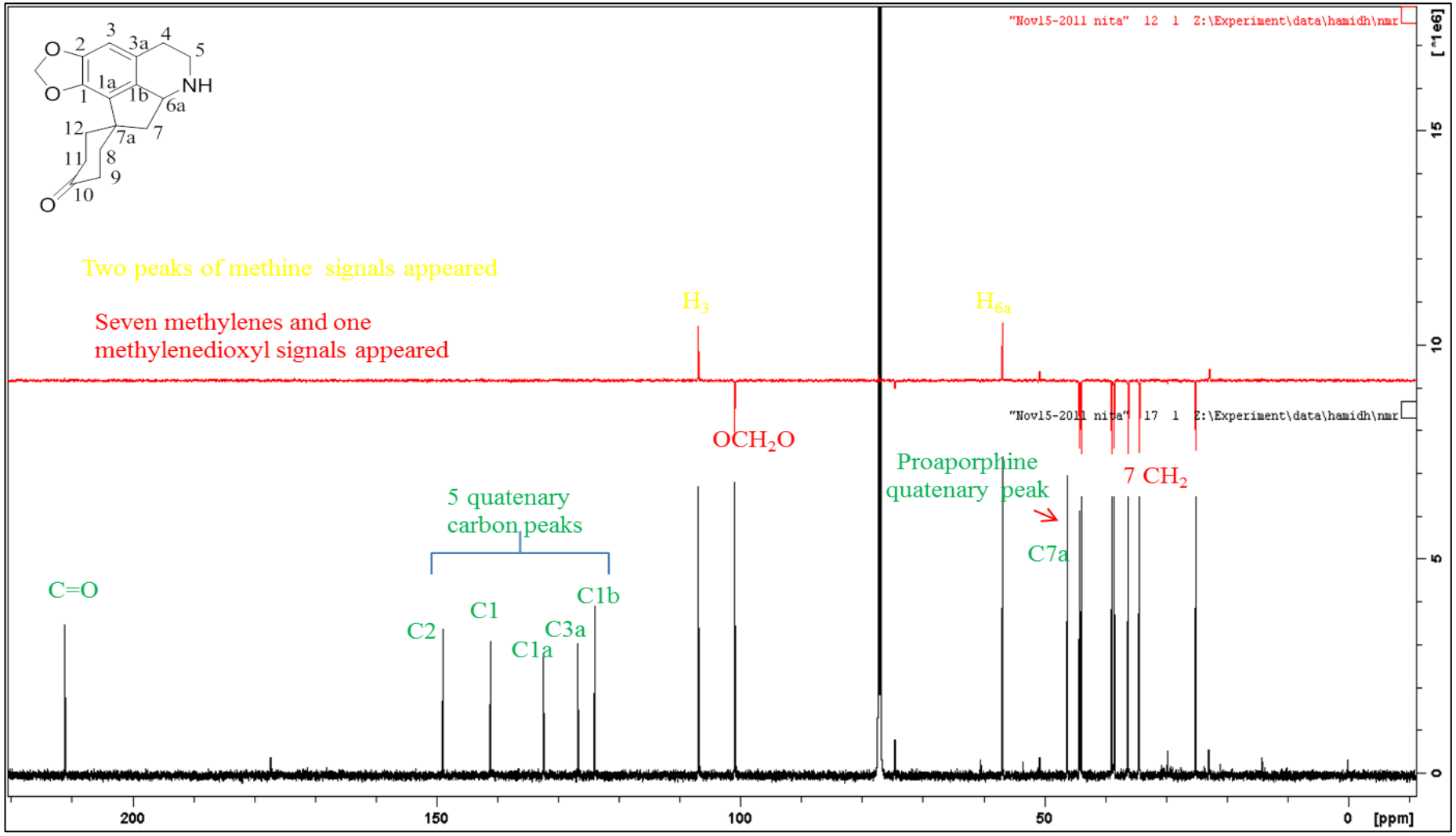


Figure 4.12: DEPT 135 and ^{13}C- NMR spectrum of litsericinone (**55**)

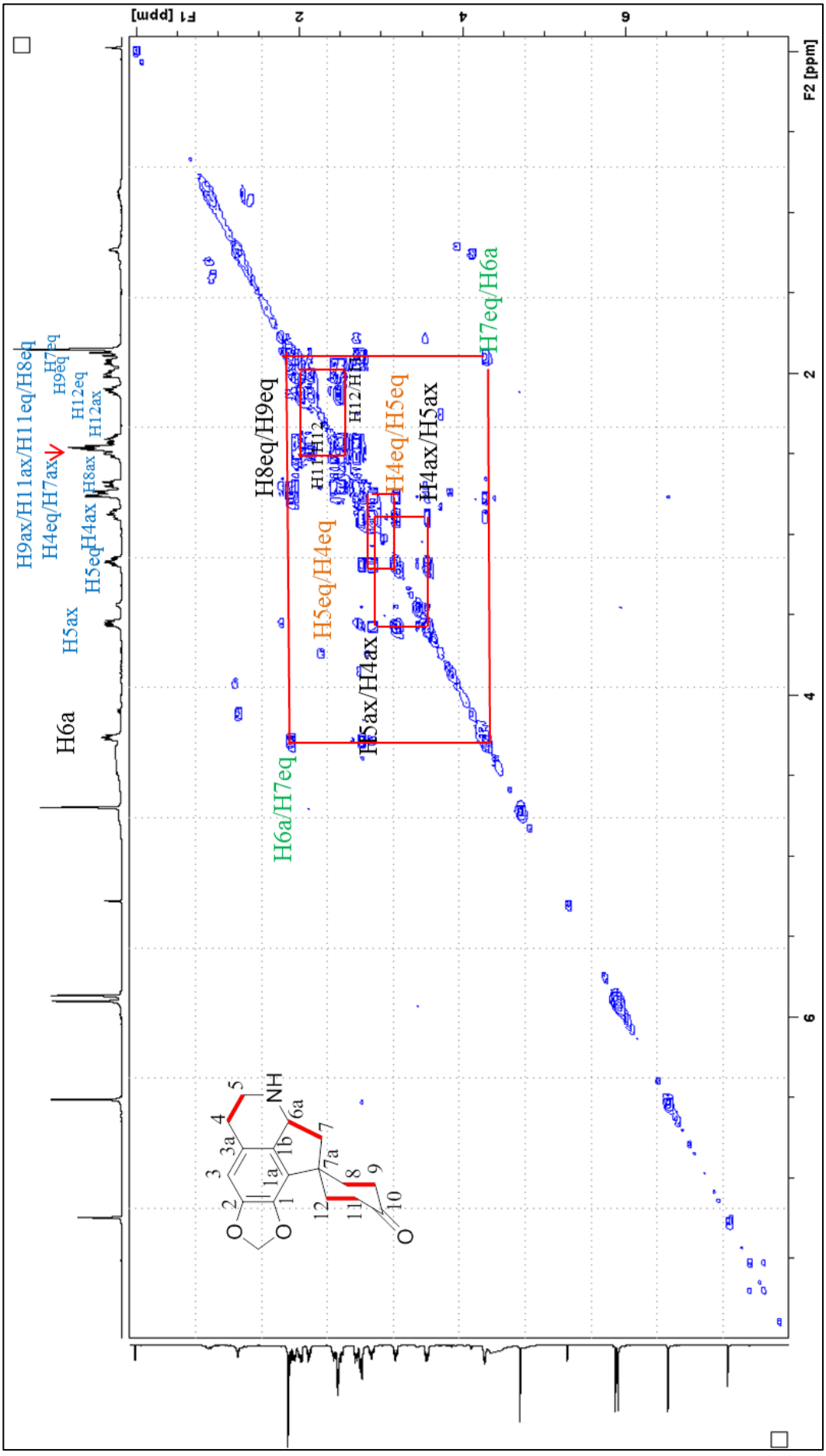


Figure 4.13: ^{1}H- ^{1}H COSY- NMR spectrum of litsericinone (**55**)

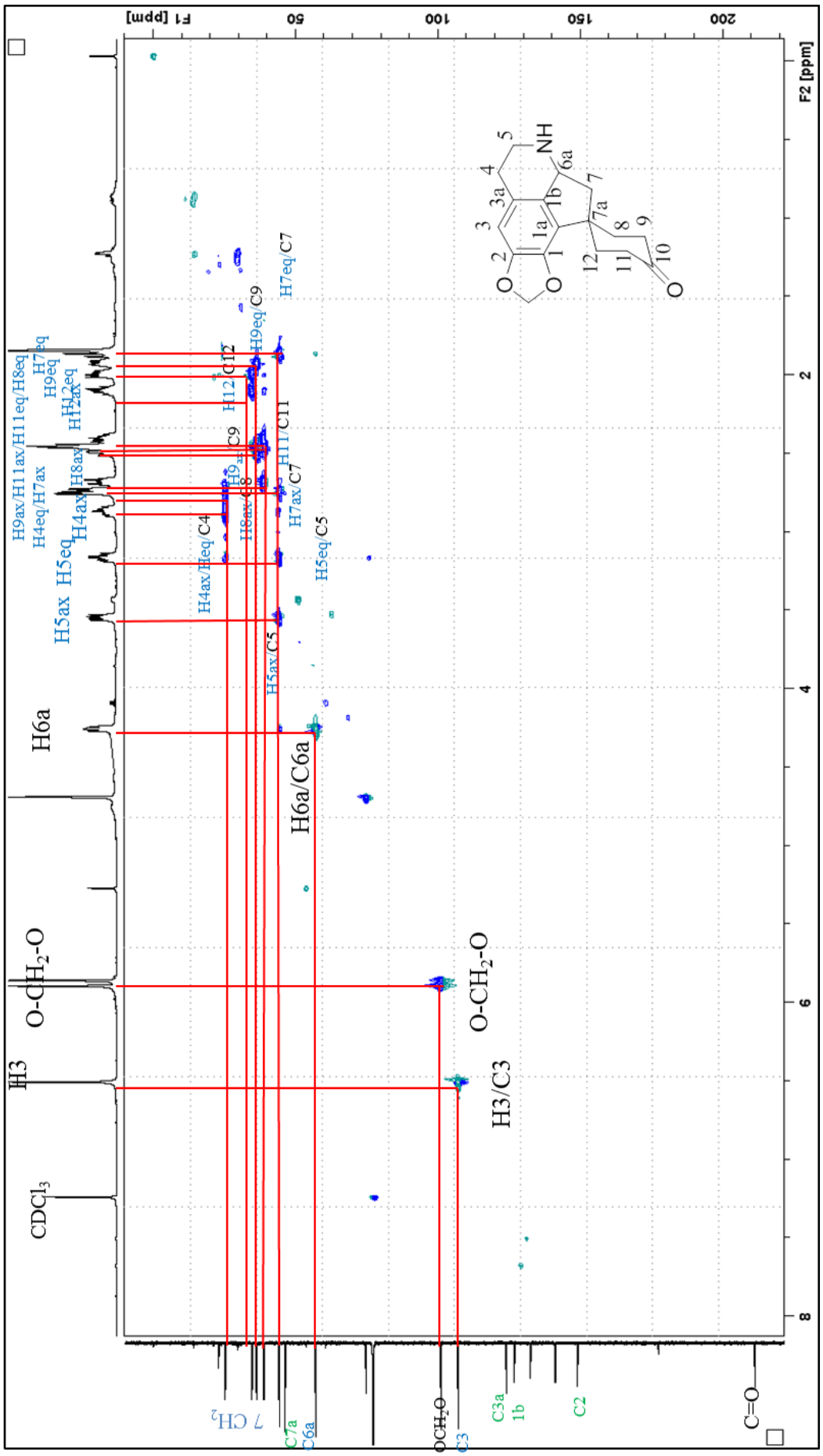


Figure 4.14: ^{1}H- ^{13}C HSQC- NMR spectrum of litsericinone (**55**)

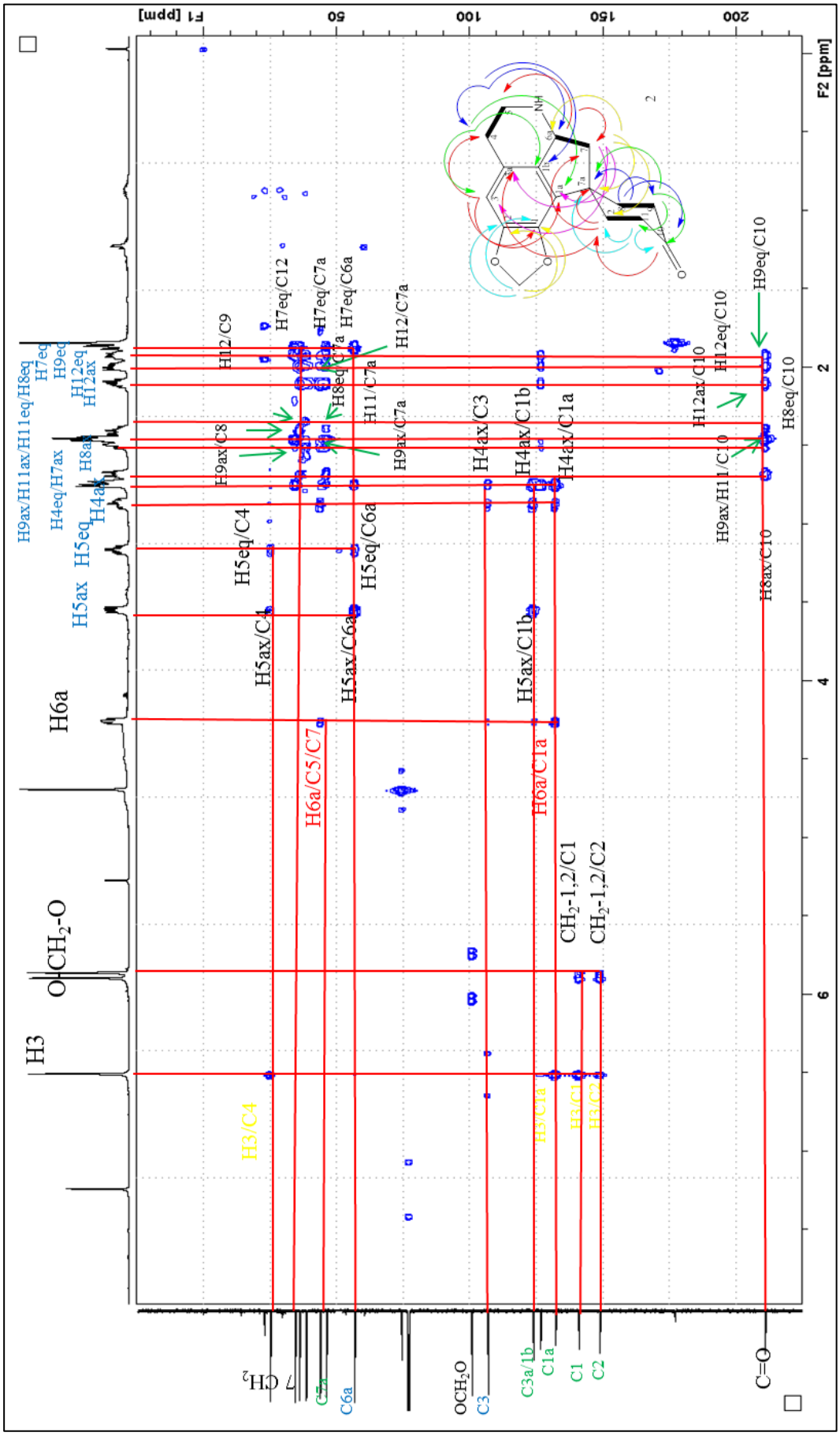
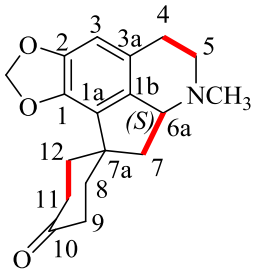


Figure 4.15: ^{1}H- ^{13}C HMBC- NMR spectrum of litsericinone (**55**)

4.2.3 8,9,11,12-tetrahydromecambrine (56)



56

8,9,11,12-Tetrahydromecambrine (**56**) was isolated as yellow amorphous solid, $[\alpha]^{29}_{D}$ = −25°(c = 0.5, $CHCl_3$). The LCMS-IT-TOF mass spectrum (Figure 4.16) of this proaporphine type of alkaloid showed a $[M+H]^+$ peak at *m/z* = 300.1596 which correlated to the molecular formula $C_{18}H_{21}NO_3$ (calcd. for $C_{18}H_{22}NO_3$, 300.1521). The UV spectrum showed an absorption peak at 203 nm. The IR spectrum (Figure 4.17) showed a very significant carbonyl absorption peak at 1712 cm^{-1} due to C=O stretching vibrations. The presence of the methylenedioxyl group was proven by its characteristic absorption peaks at 1254 and 944 cm^{-1}, which indicate the asymmetric C-O-C stretching.

The ^{1}H-NMR spectrum (Figure 4.18) displayed a pair of doublet peaks at δ 5.90 (*d*, *J* = 1.24 Hz) and 5.86 (*d*, *J* = 1.24 Hz) that correspond to the methylenedioxyl group at positions C-1 and C-2. This value, *J* = 1.2 Hz is geminal coupling constant of methylenedioxyl protons support the previous literature data (Charles *et al.*, 1987; Mukhtar *et al.*, 2008) in the range of 1.1 – 1.4 Hz. An integration of ^{1}H-NMR spectrum (Figure 4.19) showed the presence of 21 protons in the molecule structure. A singlet peak appeared at δ 6.49 representing a proton attached to a benzene ring at the C-3 position. There is a strong singlet peak at δ 2.39 indicating a N-methyl group. This is the difference between compound litsericinone (**55**) and this compound (**56**). The aliphatic protons appeared between δ 1.70 - 3.30. These peaks were supported by the COSY experiment (Figure 4.22 and 4.22.1) that showed correlation peaks between H5ax/H4ax, H4ax/H5ax, H6a/H7ax, H7ax/H6a, H6a/H7eq, H7eq/H6a, H11/H12ax,

H12ax/H11, H11/H12eq and H12eq/H11. The ^{13}C-NMR spectrum (Figure 4.20) of **56** showed the presence of eighteen carbons. The carbonyl group at C-10 position resonated at δ 211.7 and the N-methyl group at δ 43.5. The quaternary carbon at the C-7a position appeared at δ 46.0. The DEPT experiment (Figure 4.21) showed the presence of seven methylene carbons (CH_2) and one methylenedioxyl group. The assignments of all carbons were established through the aid of the HSQC (Figure 4.23) and HMBC (Figure 4.24). Based from the spectral data (Table 4.4) of this new compound which showed some similarities with litsericinone (**55**), the structure of this compound was tentatively assigned the trivial name 8,9,11,12-tetrahydromecambrine (**56**). This new natural compound was published recently (Omar *et al.*, 2013).

Table 4.4. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), and HMBC spectral data of 8,9,11,12-tetrahydromecambrine (**56**) in $CDCl_3$ (δ in ppm, J in Hz).

Position	^{1}H	^{13}C	HMBC (^{2}J,^{3}J)
1	-	140.7	-
1a	-	134.3	-
1b	-	124.5	-
2	-	148.2	-
3	6.49 *s*	106.5	C1a, C1, C2,C4
3a	-	126.9	
4	2.92 (*m*) ax	27.4	C1b, C5
	2.72 (*m*) eq		C1b, C1a, C3
5	3.09 (*m*) ax	55.0	C1b, C4, NCH_3, C6a
	2.45 (*m*) eq		
6a	3.30 br *s*	65.7	-
7	2.59 (*m*) ax	44.5	C3a, C1a, C8, C7a, C6a
	1.75 (*m*) eq		C8, C12, C7a, C6a
7a	-	46.0	-
8	2.14 (*m*) ax	34.6	C3a, C12, C7
	2.02 (*m*) eq		C3a, C12, C7a
9	2.47 (*m*)	39.0	C8, C11, C7a
			C8, C11, C7a
10	-	211.7	-
11	2.70 (*m*) ax	38.6	C12, C7a
	2.43 (*m*) eq		C12, C7a
12	2.50 (*m*) ax	36.5	C8, C11, C7
	1.93 (*m*) eq		C3a, C8, C11, C7a
N-CH_3	2.39 *s*	43.5	C5, C6a
Methlenedioxy	5.90 *d* (*J*=1.24)	100.6	C1, C2
(O-CH_2-O)	5.86 *d* (*J*=1.24)		C1, C2

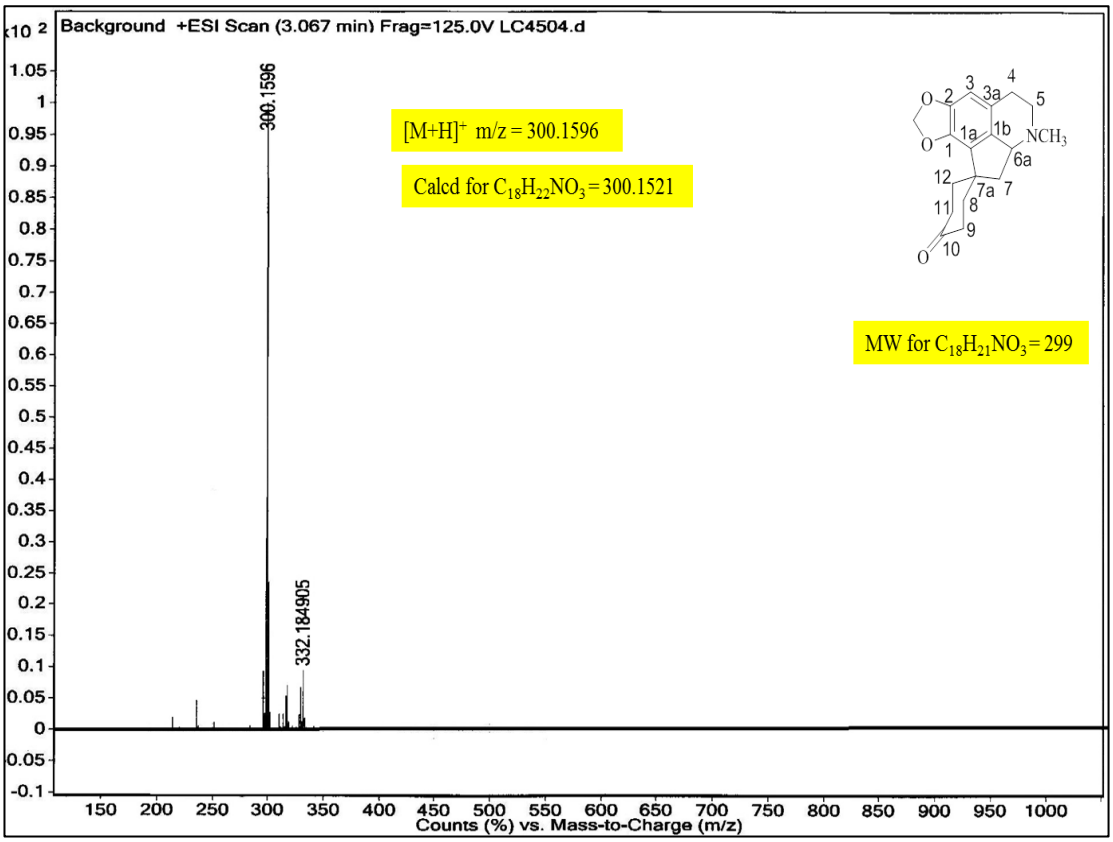


Figure 4.16: ESI- MS spectrum of 8,9,11,12-tetrahydromecambrine (**56**).

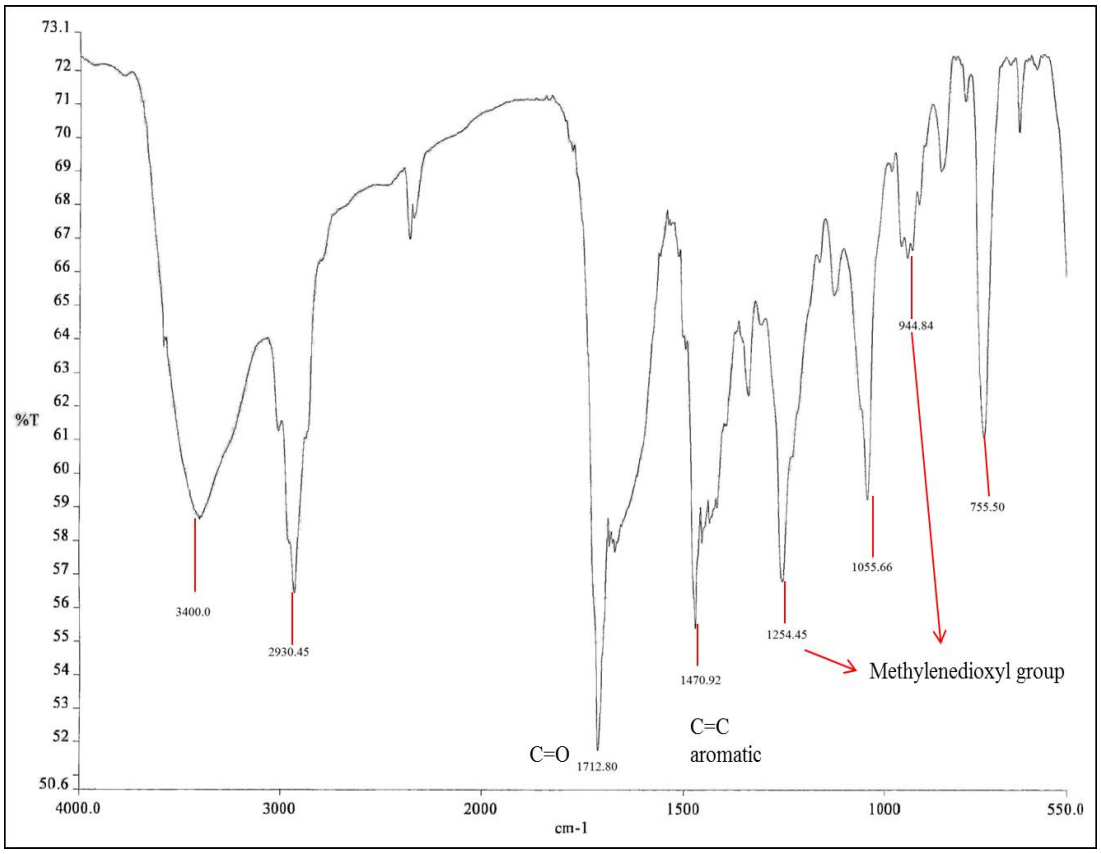


Figure 4.17: IR spectrum of 8,9,11,12-tetrahydromecambrine (**56**).

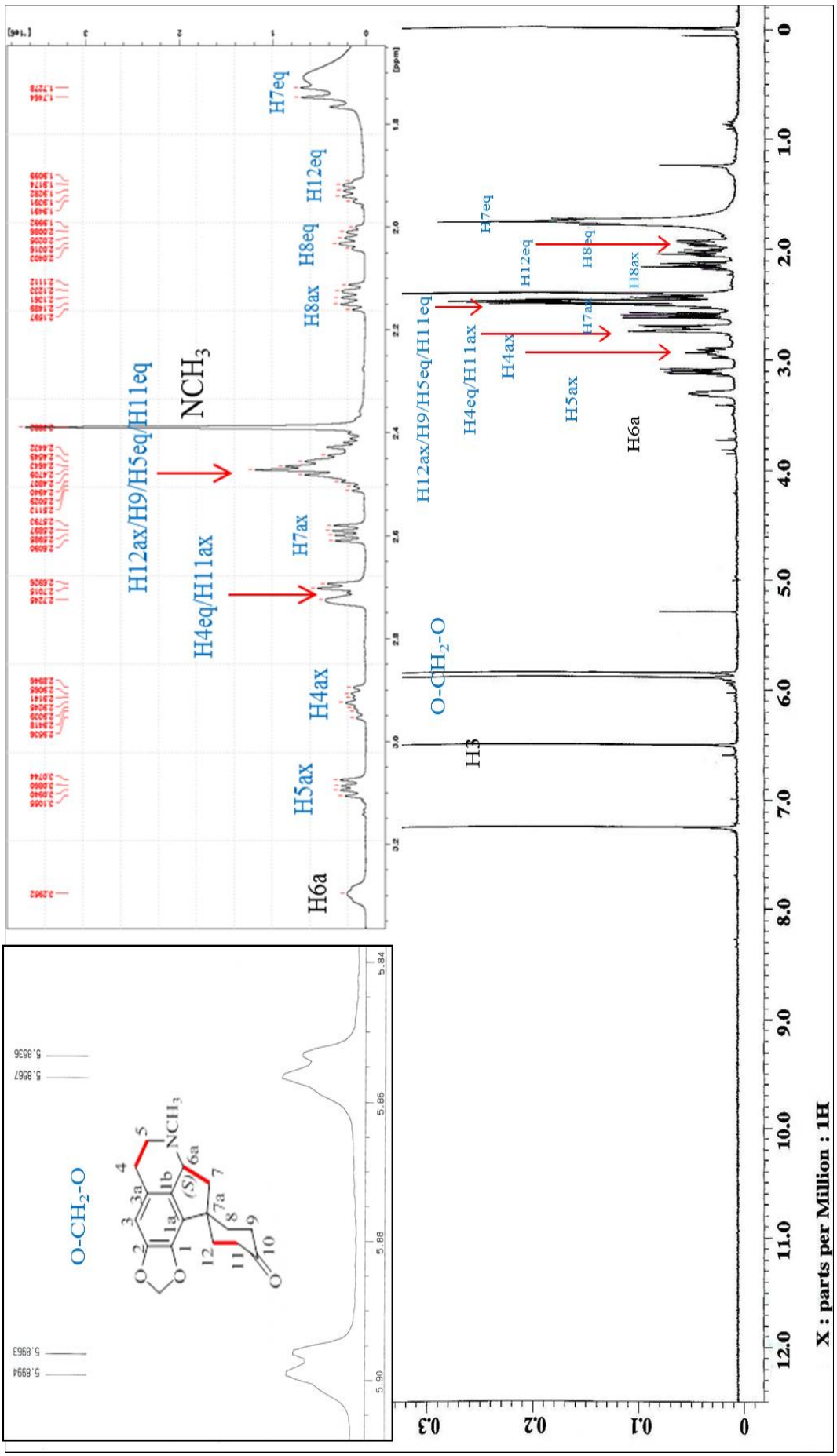


Figure 4.18: ^{1}H NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

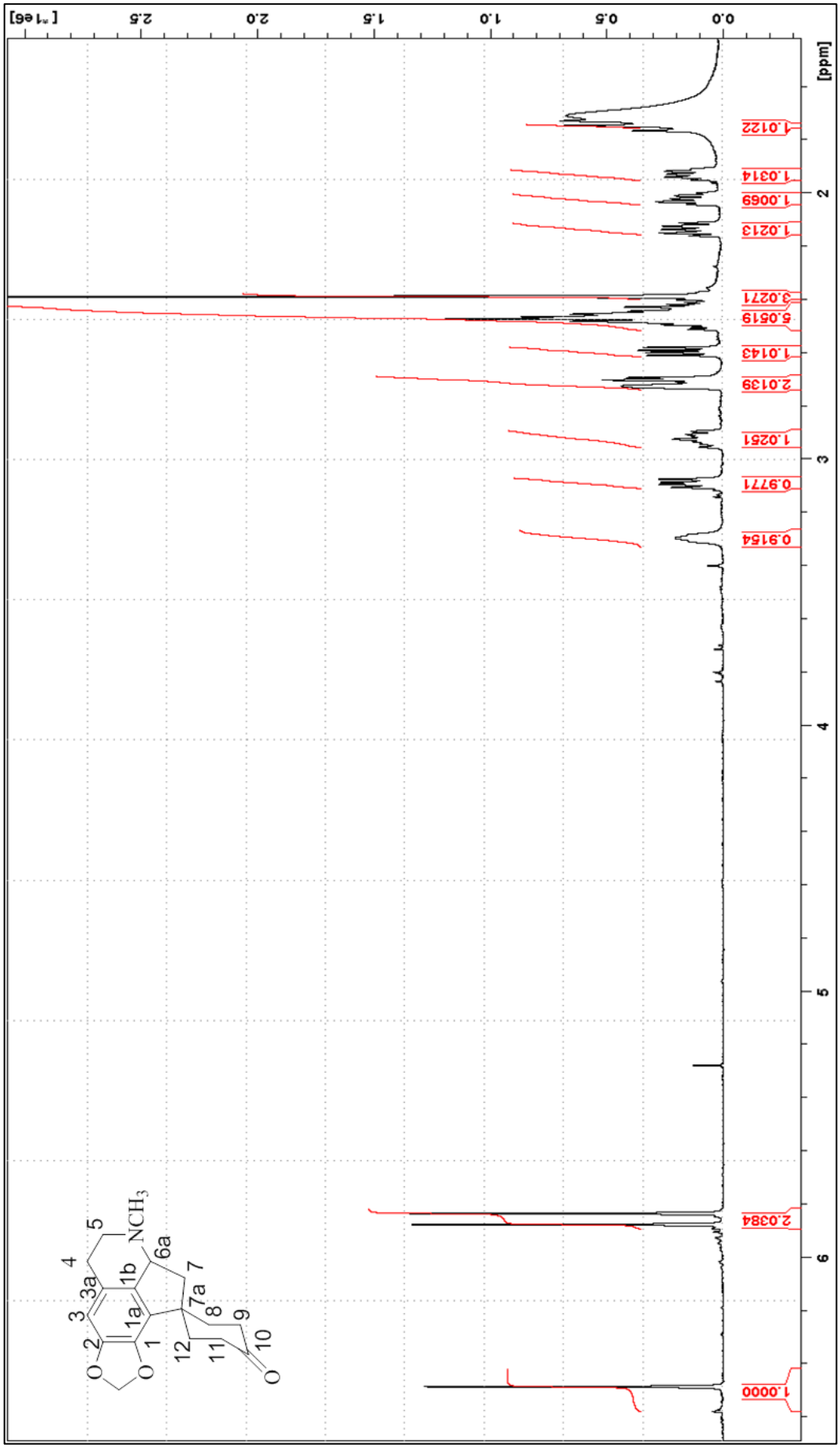


Figure 4.19: An integration ^{1}H NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**), gives the total of 21 protons.

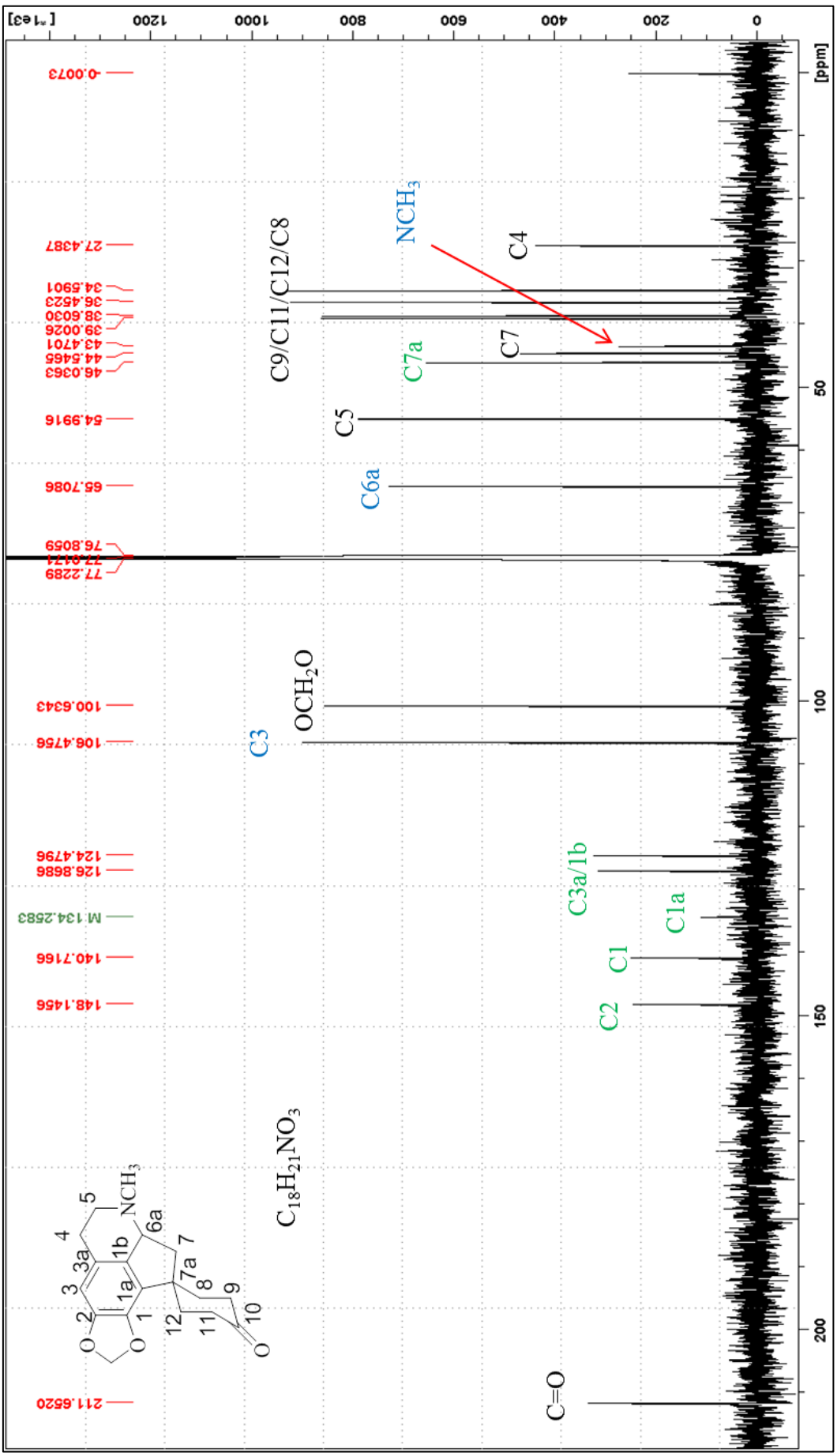


Figure 4.20: ^{13}C NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

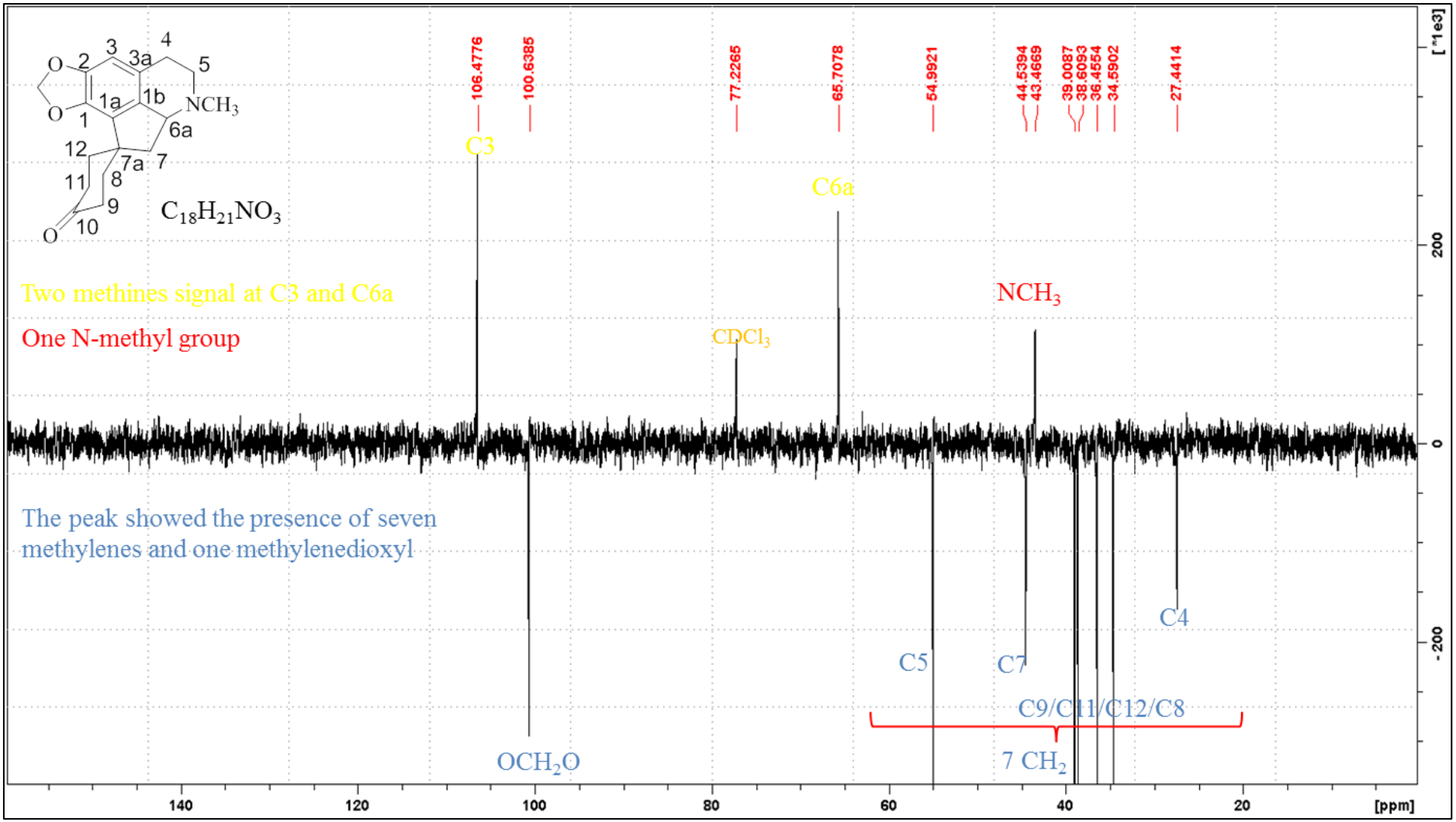


Figure 4.21: DEPT 135- NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

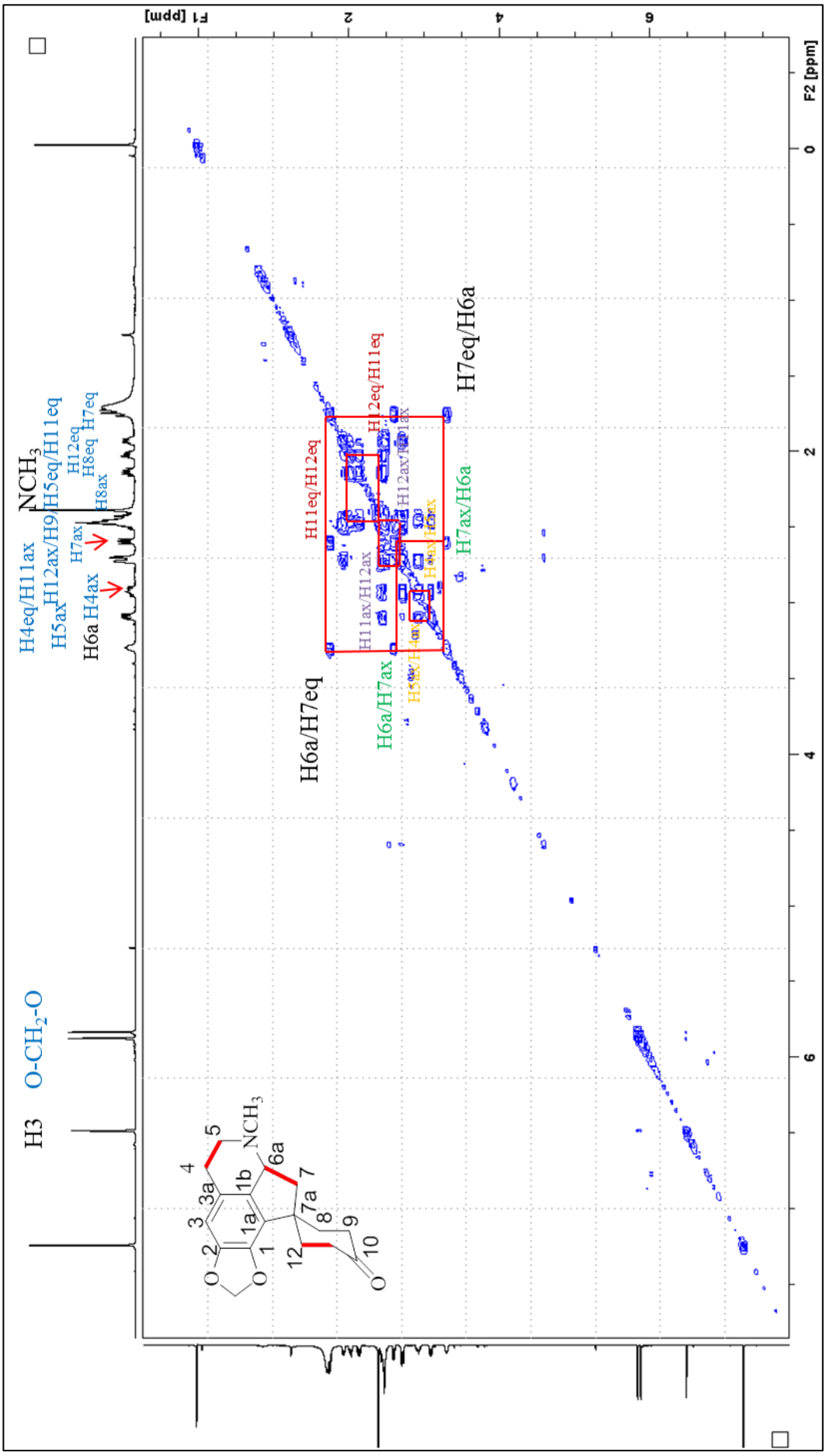


Figure 4.22: ^{1}H- ^{1}H COSY- NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

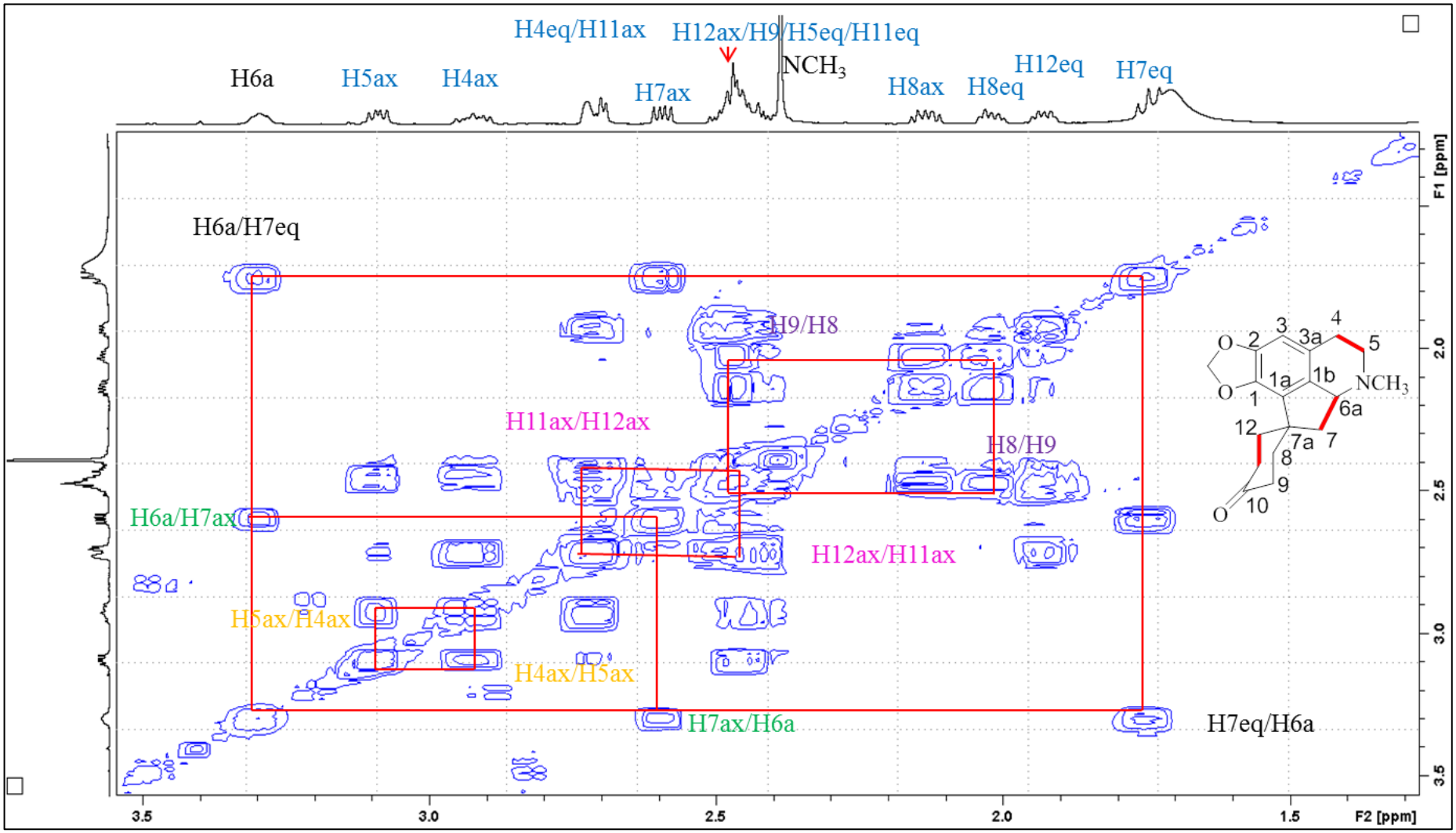


Figure 4.22.1: An expanded of ^{1}H-^{1}H COSY- NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

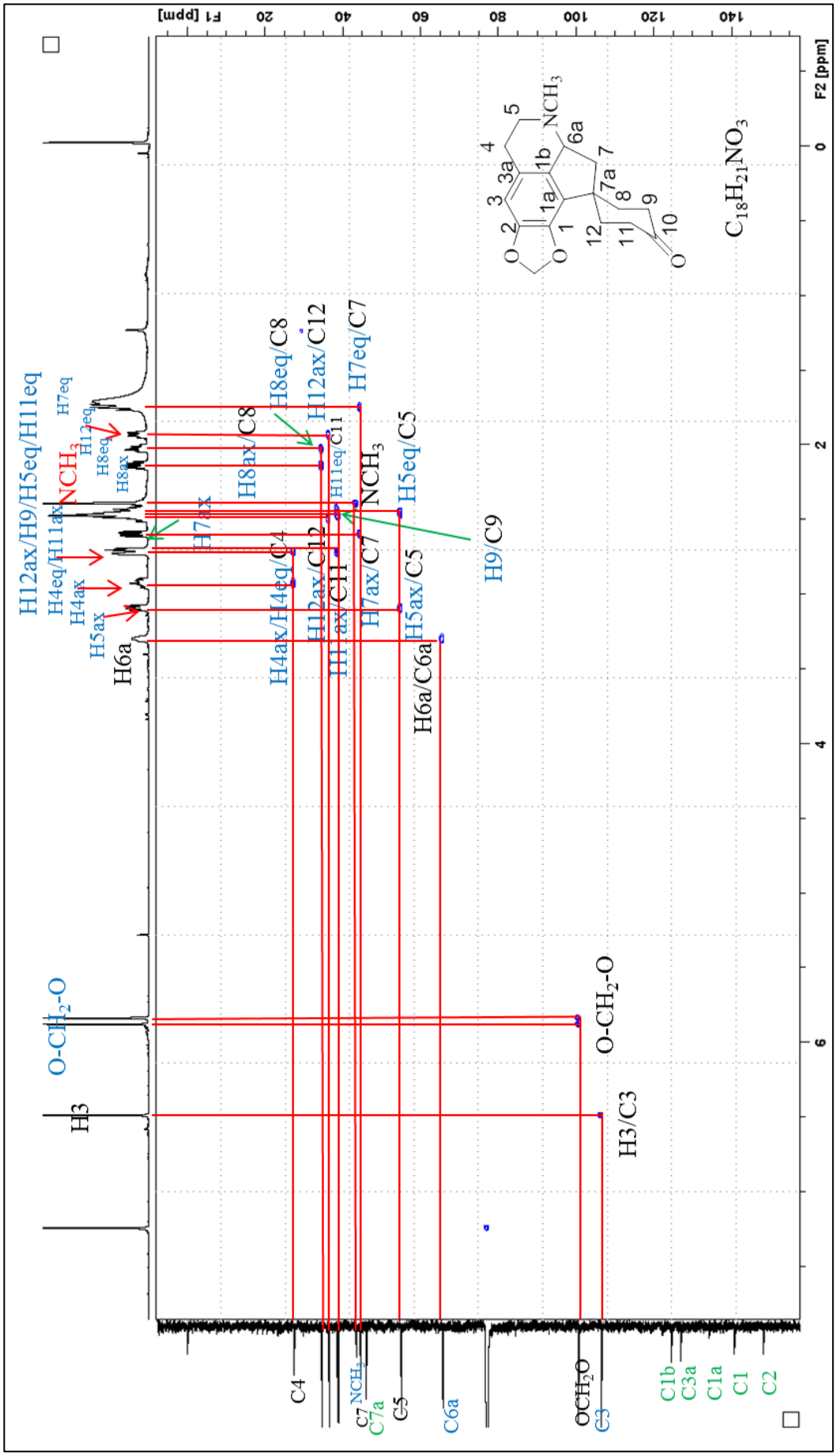


Figure 4.23: ^{1}H- ^{13}C HSQC- NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

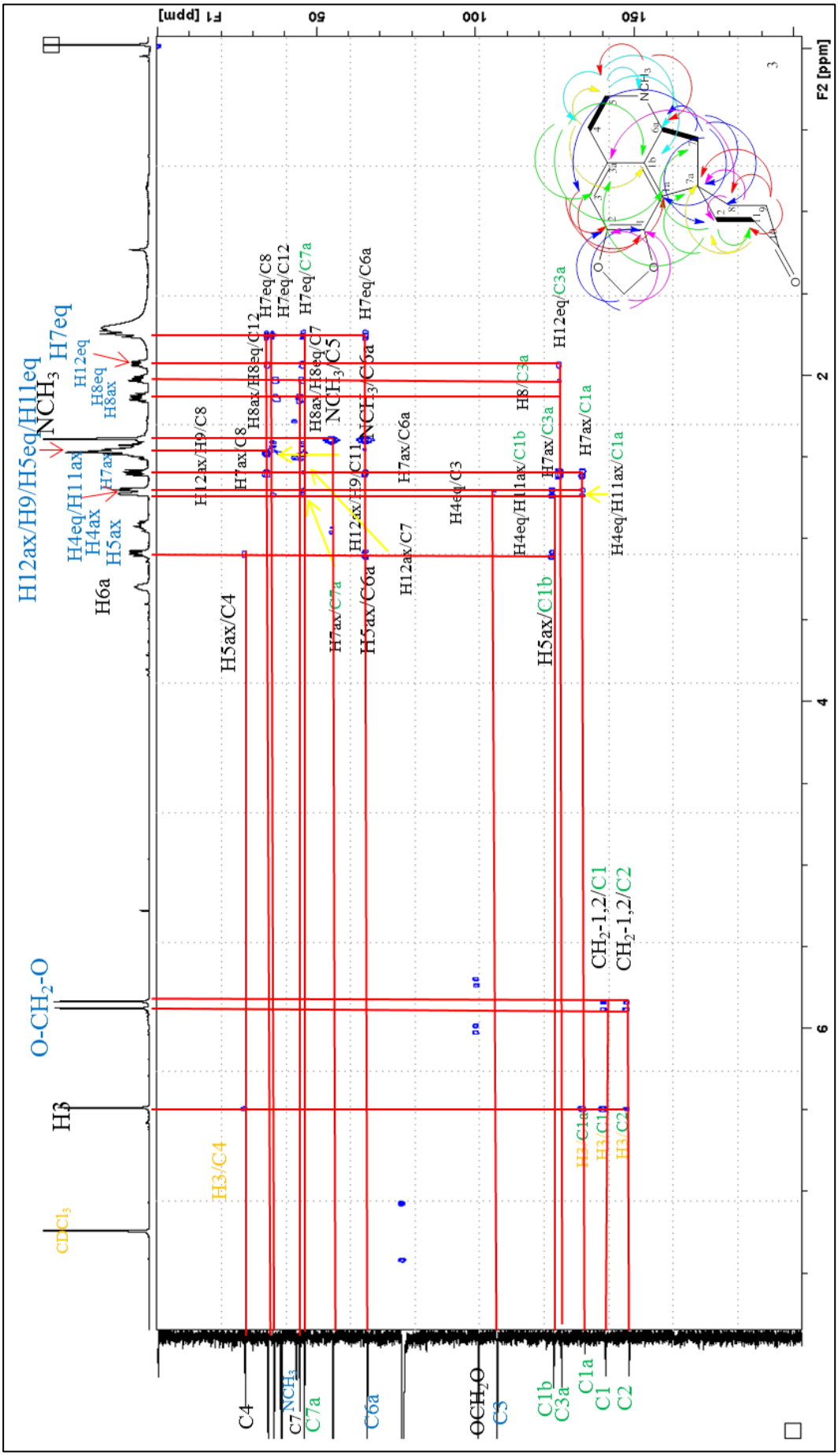
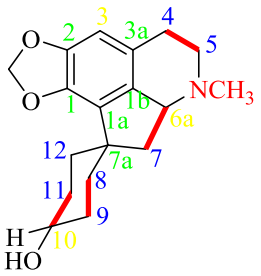


Figure 4.24: ^{1}H- ^{13}C HMBC- NMR spectrum of 8,9,11,12-tetrahydromecambrine (**56**)

4.2.4 Hexahydromecambrine A (57)



57

Alkaloid (**57**) was isolated as amorphous solid. $[\alpha]^{29}_{D}$ = +100° (c=0.5, $CHCl_3$). The mass spectrum (Figure 4.25) showed a molecular ion peak at m/z = 302.9177 $[M+H]^+$, thus suggesting a molecular formula of $C_{18}H_{23}NO_3$ (calcd for $C_{18}H_{24}NO_3$, 302.9167). The UV spectrum showed three peaks at 300, 245 and 265 nm, which indicated the existence of the conjugated system (Svatava *et al*., 1975). The IR spectrum (Figure 4.26) showed a broad absorption band at 3391 cm^{-1}, indicating the presence of a secondary hydroxyl group in the molecule (Pavia *et al.*, 1996). The methylenedioxyl group absorb at 1254 and 929 cm^{-1}, which indicate asymmetric C-O-C stretching. There was no carbonyl group present in the IR spectrum.

The ^{1}H-NMR spectra (Figure 4.27) for this alkaloid **57** showed a pair of doublet peaks at δ 5.90 ppm (*d*, *J* = 1.20 Hz) and δ 5.86 ppm (*d*, *J* = 1.20 Hz) that correspond to methylenedioxyl group at position C-1 and C-2. A sharp singlet peak at 6.47 ppm belongs to H-3 on the benzene ring. The aliphatic protons of ring D resonated as multiplets between δ 1.25 - 2.45. H-6a resonated as multiplet at δ 3.26. A strong peak at δ 2.39 appeared as a sharp peak was assigned for N-methyl group. H-10, which is in proximity to a hydroxyl group resonated further upfield at δ 4.00 as a broad multiplet suggesting the possibility of an axial configuration. Interestingly, from the COSY spectra (Figure 4.29), H-10 was determined at be pseudo-axial by the correlations H-10

(δ 4.00) with H-8_{ax} (δ 2.03) and H-11 (δ 1.75). In the case of pseudo-equatorial hydroxyl group, H-10 will resonate at around δ 4.26 – 4.39 (Ricca & Casagrande, 1977).

The ^{13}C-NMR spectrum (Figure 4.28) of **57** showed the presence of eighteen carbons, which is in agreement with the molecular formula $C_{18}H_{23}NO_3$. The DEPT spectrum (Figure 4.28), showed the presence of one N-methyl, eight methylenes and three methines. The ^{13}C-NMR and DEPT spectrum (Figure 4.28), gave six quaternary carbons signals. The methylenedioxyl group resonated at δ 100.5. The characteristic proaporphine quaternary spirocarbon peak C-7a appeared at δ 46.6. Apparently, C-10 resonated at δ 67.1 further strengthen the hypothesis of H-10 being axial. The complete assignments of all protons and carbons were aided by the HSQC (Figure 4.30) and HMBC correlation (Figure 4.31). HMBC, HSQC and COSY data were recorded in Table 4.5, since these data are not available in the literature. The structure of **57** resembled to structure of **56** with the exceptional of the carbonyl group in **56** was reduced to hydroxyl group in **57**.

Comparison with the literature values in Table 4.5 (Mukhtar, 2003), confirmed that alkaloid **57** is hexahydromecambrine A. Compound **57** was isolated for the first time from *Phoebe grandis*. Compound **57** has also been isolated from *Phoebe scortechinii* (Mukhtar *et al*., 2008) and was previously synthesized by Nakasato (Nakasato & Asada, 1966).

Table 4.5: ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz) and 2D (HMBC and HSQC) NMR data of hexahydromecambrine A (**57**) in $CDCl_3$ and the literature data.

	57 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	148.1	-	-	-	148.0
1a	-	129.0	-	-	-	128.8
1b	-	131.0	-	-	-	135.8
2	-	140.8	-	-	-	140.4
3	6.46 (*s*)	105.9	C_1, C_2,C_4	H_3	6.45 *s*	105.9
3a	-	124.0	-	-	-	124.1
4	2.93 (*m*) ax	27.3	C_5	$2H_4$	2.61 *m*	27.5
	2.71 (*m*) eq		C_{3a}		2.55 *m*	
5	3.11 (*m*) ax	54.9	C_{3a}, C_{6a}	$2H_5$	3.38 *m*	55.1
	2.46 (*m*) eq				3.07 *m*	
6a	3.26 (*m*)	65.7	-	H_{6a}	3.18 *dd* (6.3, 10.3)	66.0
7	2.44 (*m*) ax	44.3	C_{1a}	$2H_7$	2.55 *m*	42.9
	1.58 (*m*) eq		C_{12}, C_9		1.45 *m*	
7a	-	46.6	-	-	-	46.0
8	2.03 (*m*) ax	30.2	C_{7a}, C_{10}	$2H_8$	2.25 *m*	35.0
	1.54 (*m*) eq		C_{12}, C_{7a}, C_{10}		1.60 *m*	
9	2.41 (*m*) ax	31.7	-	$2H_9$	1.75 *m*	32.1
	1.46 (*m*) eq		C_8, C_{7a}, C_{10}			
10	4.00 br, *m*	67.1	-	H_{10}	3.71 br, *m*	70.4
11	1.75 (*m*)	31.0	C_{12}, C_{7a}, C_{10}	$2H_{11}$	1.94 *m*	33.1
12	1.25 (*m*)	29.7	-	$2H_{12}$	1.74 *m*	32.0
					1.76 *m*	
N-CH_3	2.39 (*s*)	43.2	C_5, C_{6a}	3H	2.35 *s*	43.5
OCH_2O	5.90 (*d*, *J* = 1.2)	100.5	C_1, C_2	2H	5.87 (*d*, 1.3)	100.6
	5.86 (*d*, *J* = 1.2)		C_1, C_2		5.82 (*d*, 1.3)	

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.
* (Mukhtar, 2003)

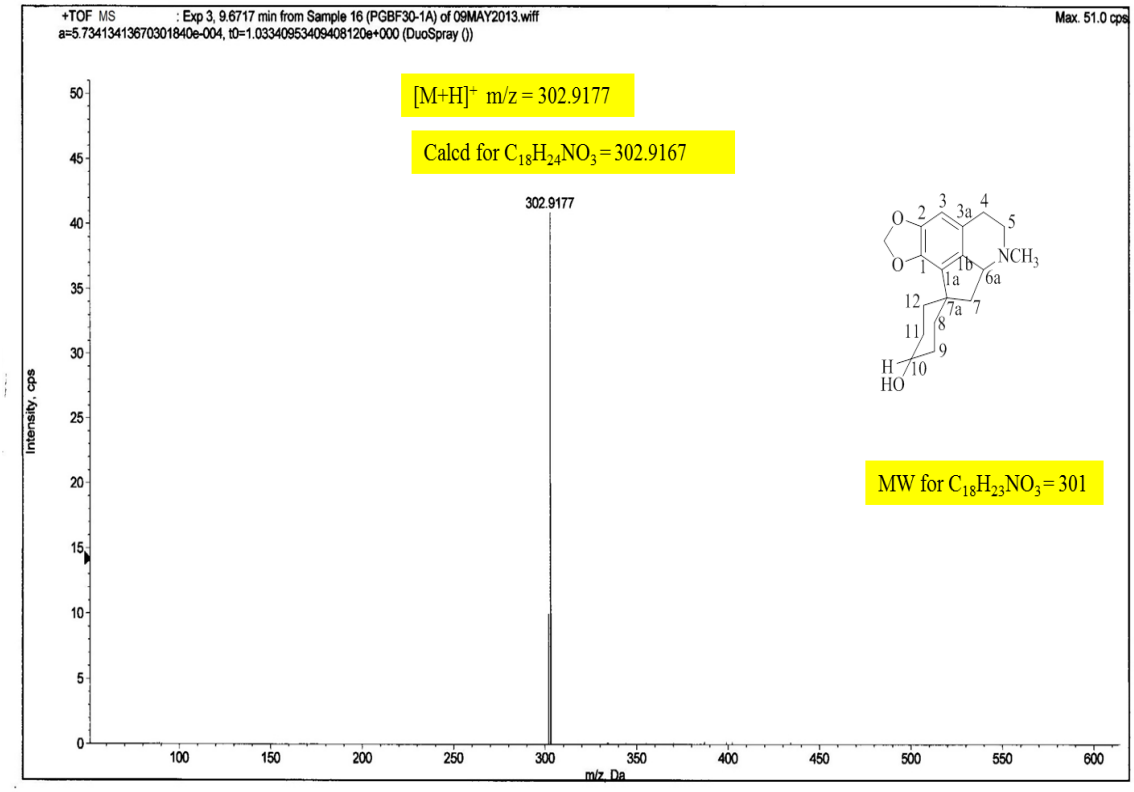


Figure 4.25: LCMS Triple TOF-MS spectrum of hexahydromecambrine A (**57**)

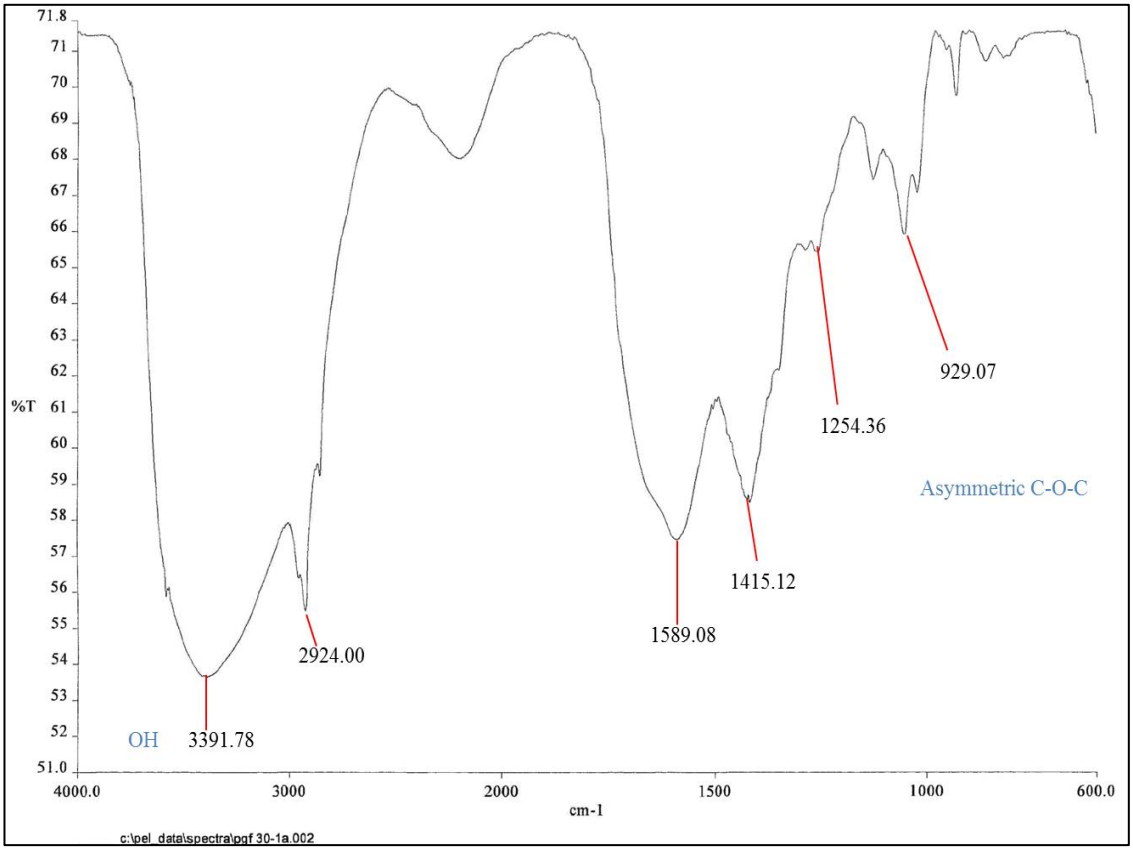


Figure 4.26: IR spectrum of hexahydromecambrine A (**57**).

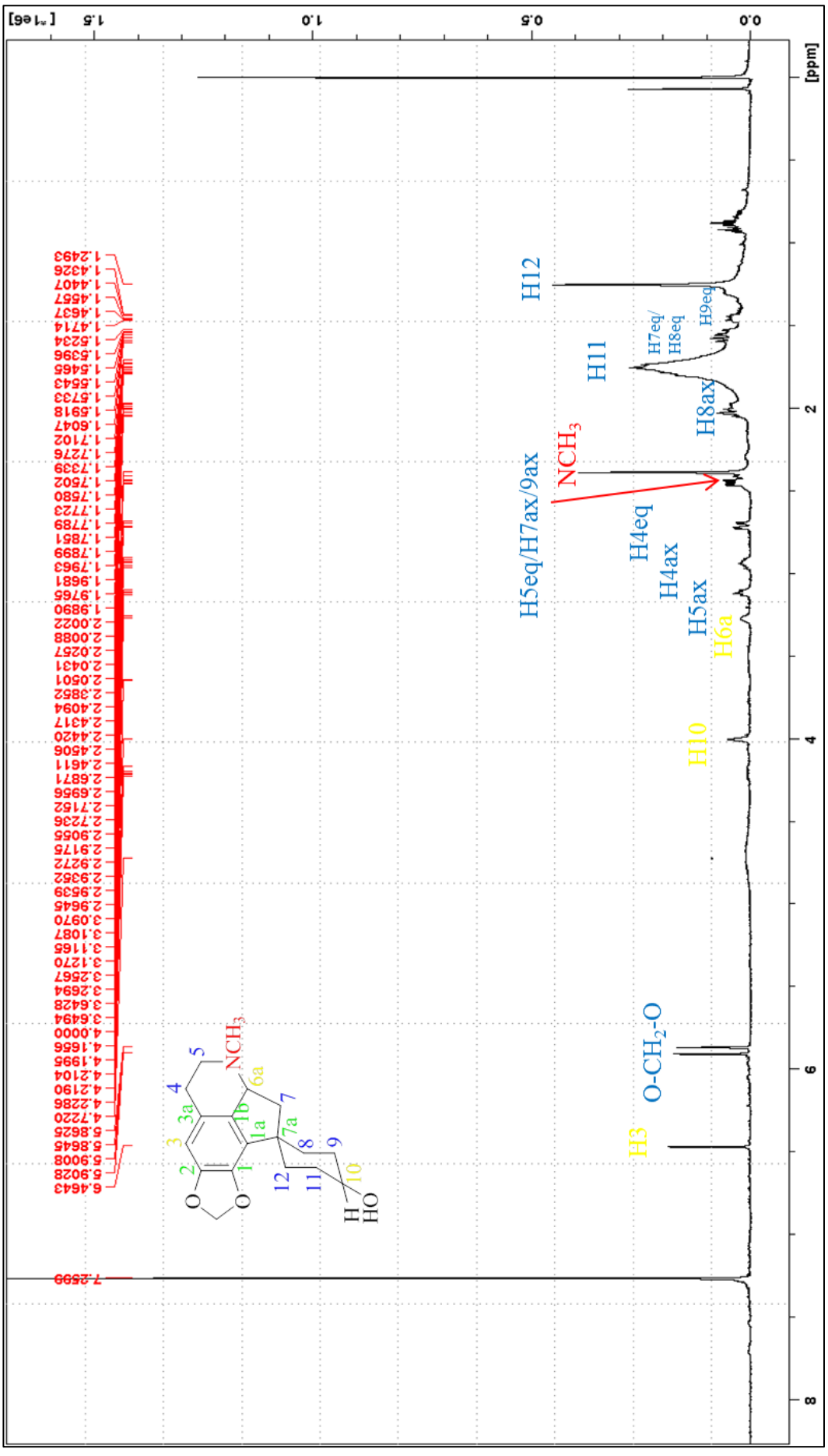


Figure 4.27: ^{1}H NMR spectrum of hexahydromecambrine (**57**)

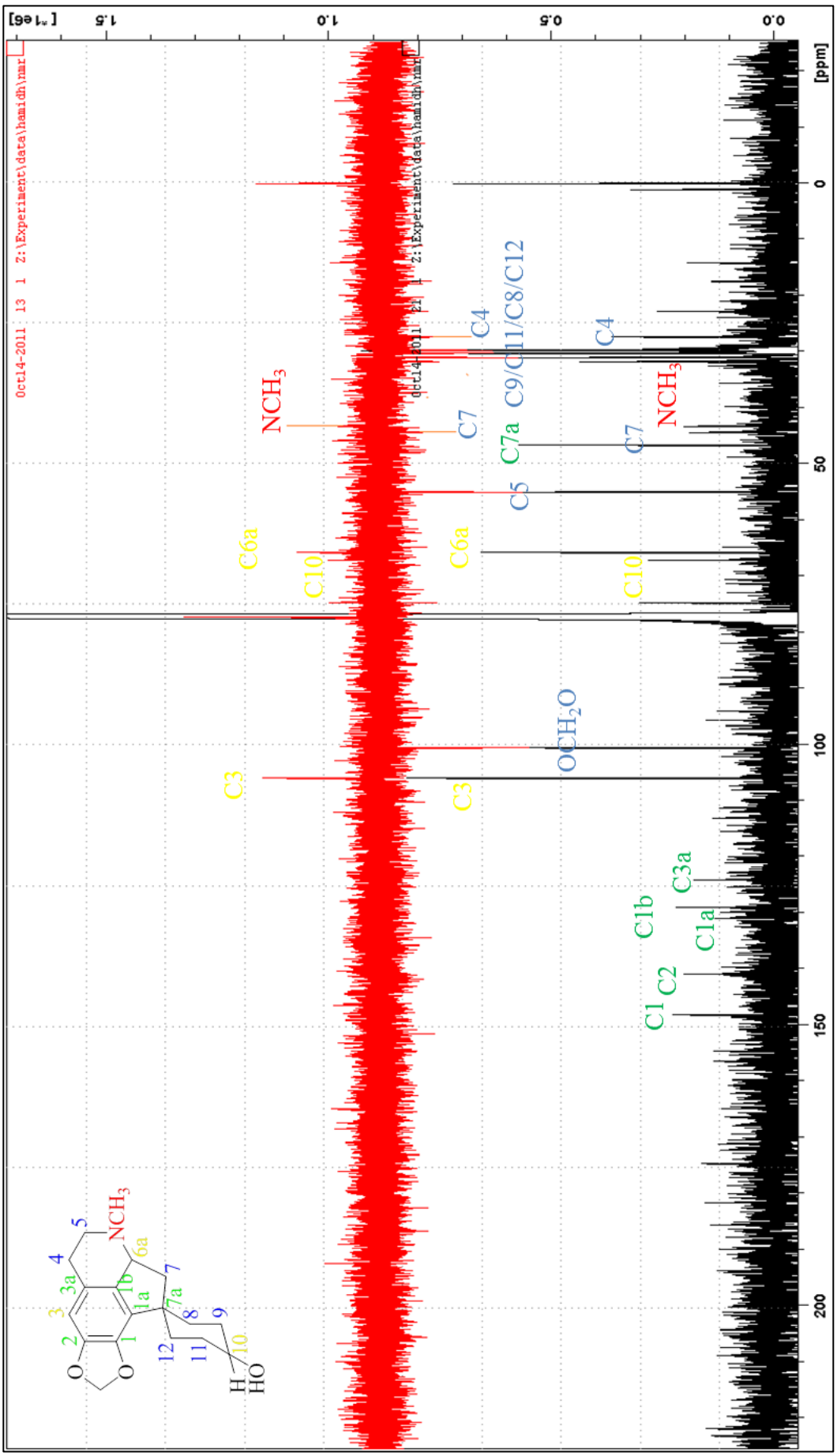


Figure 4.28: ^{13}C and DEPT 135- NMR spectrum of hexahydromecambrine A (**57**)

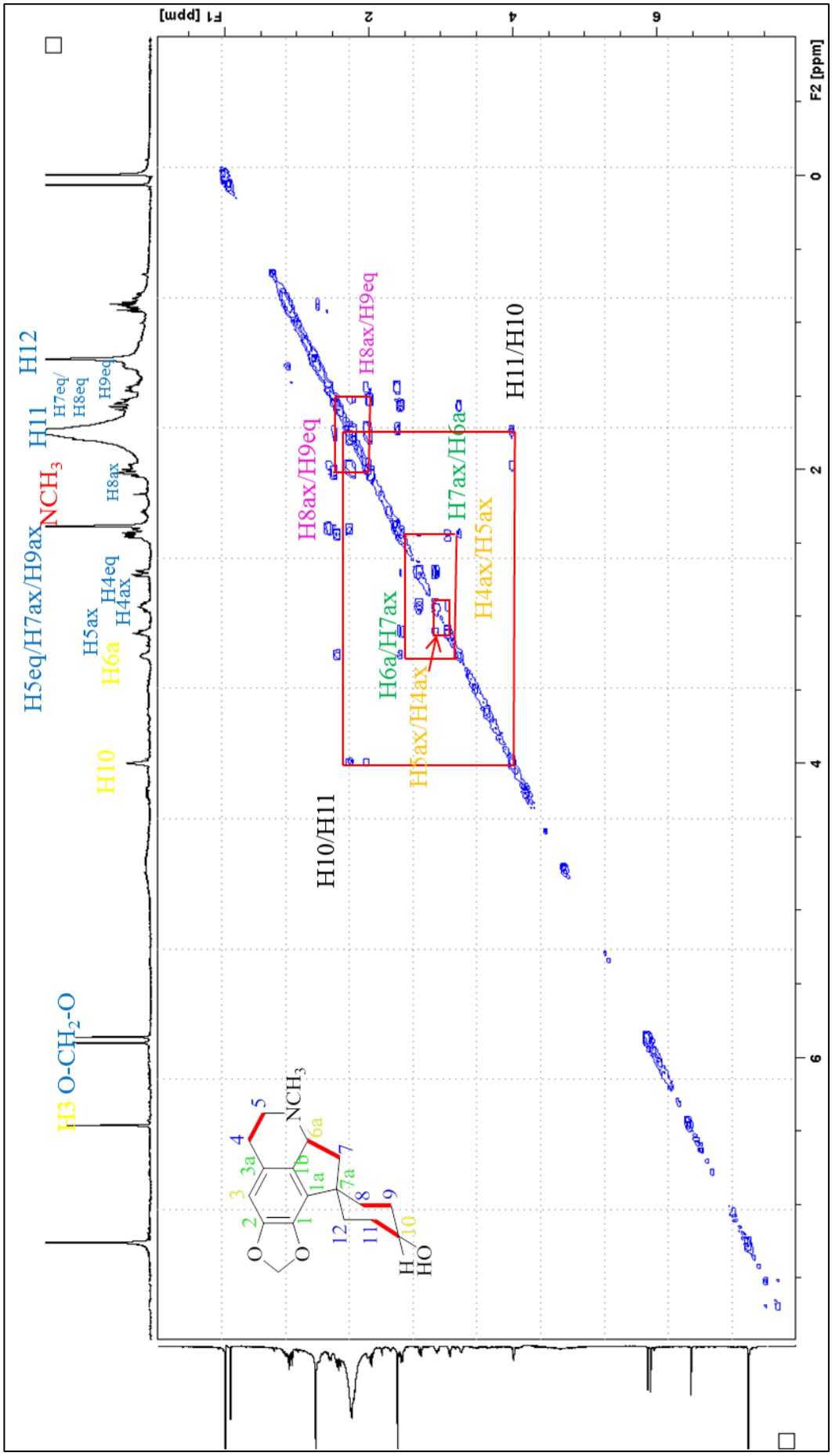


Figure 4.29: ^{1}H- ^{1}H COSY- NMR spectrum of hexahydromecambrine A (**57**)

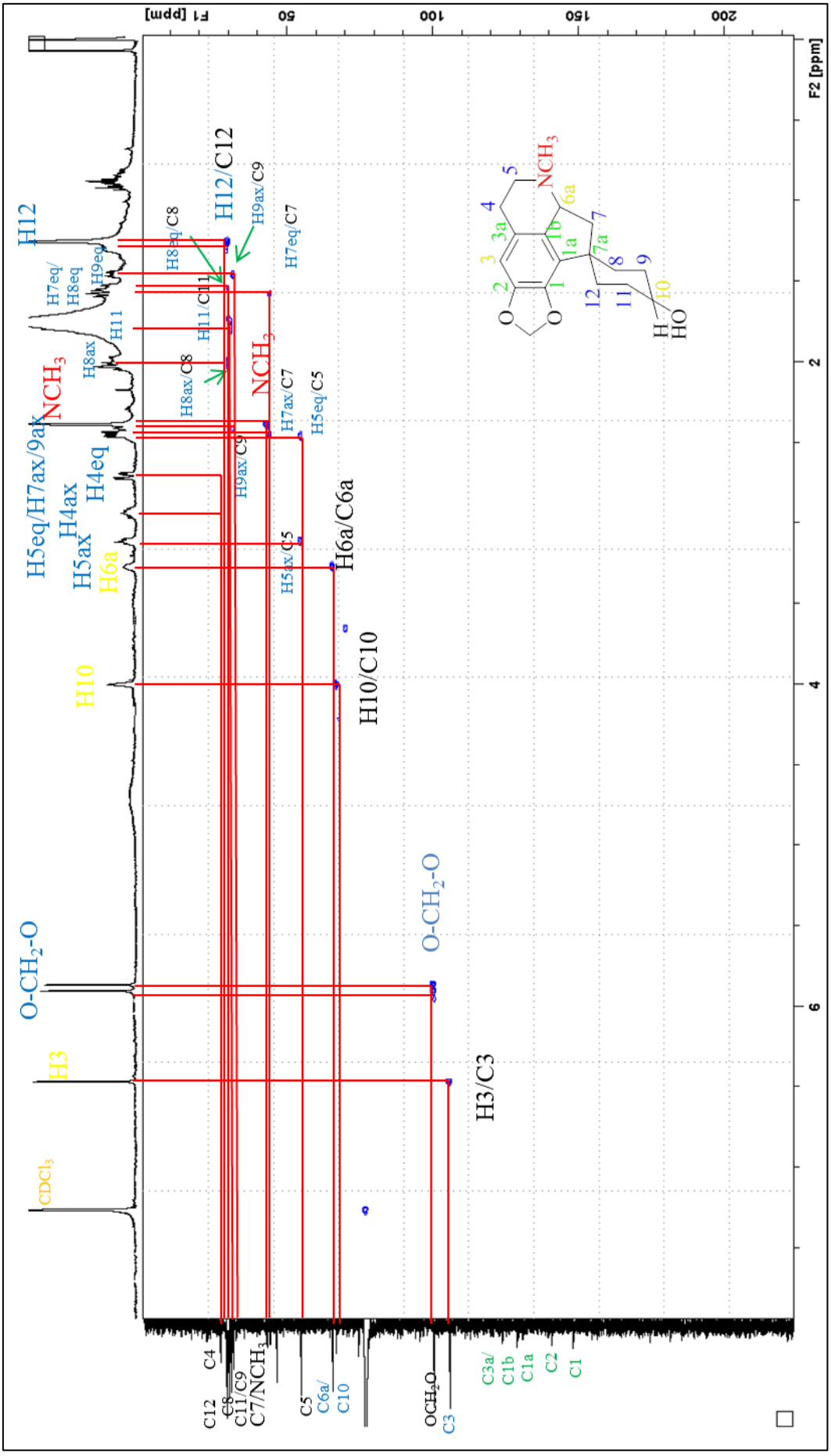


Figure 4.30: ^{1}H- ^{13}C HSQC- NMR spectrum of hexahydromecambrine A (**57**)

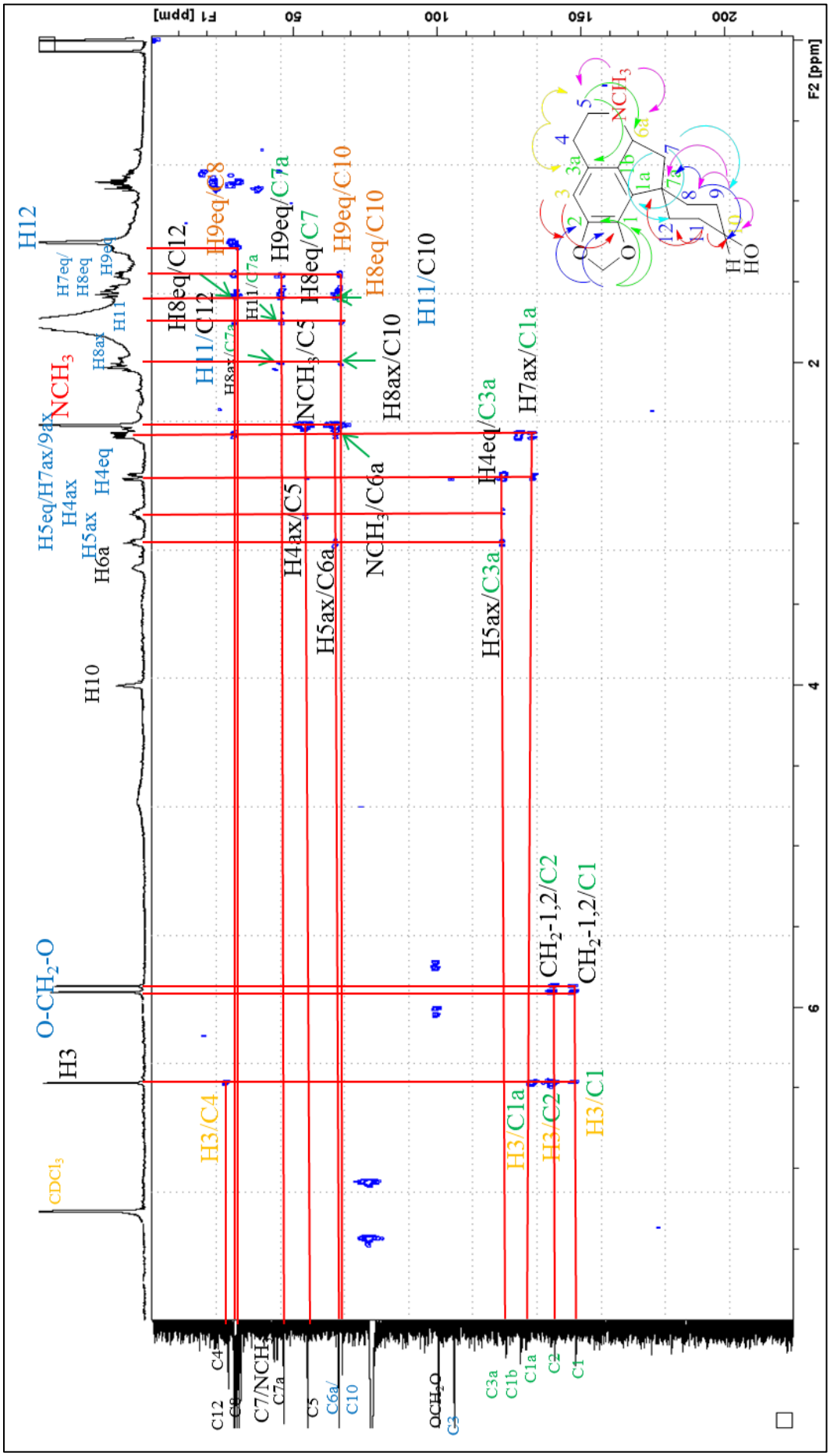
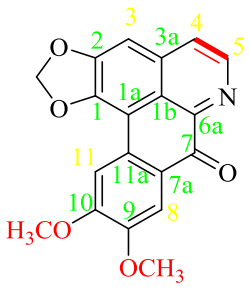


Figure 4.31: ^{1}H- ^{13}C HMBC- NMR spectrum of hexahydromecambrine A (**57**)

4.2.5 Dicentrinone (58)



58

Compound **58** was obtained as fine yellow needles. The mass spectrum (Figure 4.32) showed a molecular ion peak at m/z = 336.0863 $[M+H]^+$, thus suggesting a molecular formula of $C_{19}H_{13}NO_5$ (calcd for $C_{19}H_{14}NO_5$, 336.0827). UV spectrum of **58** exhibited peaks at 218, 257, 270, 308, 347 and 399nm which were consistent and supported the literature values at 213, 254, 271, 311, 349, and 400 nm (Zhou *et al.*, 2000). The IR spectrum (Figure 4.33) of **58** showed the absorption peak at δ 1276 and 995 cm^{-1} due to the presence of methylenedioxy group at C1 and C2. The strong peak at 1725 cm^{-1} showed the presence of the C=O carbonyl group at C7. The CH-aromatic stretching peaks appeared at δ 2916 and 2849 cm^{-1}.

The ^{1}H-NMR spectra (Figure 4.34) showed the two strong singlet peaks indicated the presence of two CH_3O- groups at position C9 and C10 [δ_H 4.00 and δ_C 55.1; δ_H 4.06 and δ_C 55.3], one methylenedioxy group [δ_H 6.34 (2H, s) and δ_C 101.4], three isolated singlet aromatic protons with two protons at para position with respect to each other [δ_{H11} 7.99 and δ_C 107.8; δ_{H8} 7.97 and δ_C 108.6; δ_{H3} 7.11 and δ_C 101.7], and two *ortho*-coupled aromatic protons [δ_{H5} 8.86 (1H, d, *J*=5.2) and δ_C 143.8; δ_{H4} 7.72 (1H, d, *J*=5.2) and δ_C 123.0]. The respective position of the protons at C-4 and C-5 were confirmed by ^{1}H-^{1}H correlations in the COSY spectra (Figure 4.36).

The ^{13}C and DEPT 135-NMR (Figure 4.35) revealed the presence of 19 signals corresponding to 19 carbons in the molecules. The most downfield signals was assigned as one carbon carbonyl δ_C 180.3, five methine carbons [δ_C 143.8, 108.6, 107.8, 101.7, 143.8, 123.0] and eleven quaternary carbons. Meanwhile, the methoxyl carbon signals appeared at δ_C 55.1 and 55.3. The peak at δ_C 101.4 indicated the presence of methylenedioxy group in the molecule which was confirmed by DEPT spectrum (Figure 4.35).

The ^{1}H-^{13}C direct correlations were determined by using HSQC spectrum (Figure 4.37) and the results were supported by the other data. The structure was finally confirmed by ^{1}H-^{13}C long range correlations in the HMBC spectrum (Figure 4.38). These collected data, together with comparison with data from the literature, established the structure of compound **58** as that of the known oxoaporphine alkaloid dicentrinone (**58**) (Zhou *et al*., 2000). Because its NMR assignments have not previously been complete reported in the literature, we assigned its ^{1}H and ^{13}C NMR spectra unambiguously by the long range correlations in its HMBC and HMQC spectra as shown in Table 4.6. Interestingly, it was isolated for the first time from *Phoebe grandis*.

Table 4.6: ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz) and 2D (HMBC and HSQC) NMR data of dicentrinone, (**58**) in $CDCl_3$ and the literature data.

	58 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	150.6	-	-	-	152.2
1a	-	107.3	-	-	-	107.5
1b	-	121.6	-	-	-	122.2
2	-	146.1	-	-	-	147.6
3	7.11 (1H, *s*)	101.7	C_{1b}, C_2, C_4	H_3	6.14 (1H, *s*)	102.5
3a	-	134.6	-	-	-	136.2
4	7.72 (1H, *d*, *J*=5.2)	123.0	C_{1b}	H_4	7.54 (1H, *d*, *J*=5.2)	124.3
5	8.86 (1H, *d*, *J*=5.2)	143.8	C_{3a}, C_{6a}	H_5	8.48 (1H, *d*, *J*=5.2)	142.9
6a	-	146.0	-	-	-	143.7
7	-	180.3	-	-	-	180.4
7a	-	124.9	-	-	-	125.0
8	7.97 (1H, *s*)	108.6	C_{11a}, C_{10}, C_7	H_8	7.58 (1H, *s*)	109.1
9	-	148.5	-	-	-	149.4
10	-	152.9	-	-	-	154.1
11	7.99 (1H, *s*)	107.8	C_8, C_{7a}, C_9, C_{10}	H_{11}	7.59 (1H, *s*)	108.8
11a	-	126.8	-	-	-	127.7
9-OCH_3	4.00 (3H, *s*)	55.1	C_{10}	$3H_{9\text{-OMe}}$	3.74 (3H, *s*)	55.6
10-OCH_3	4.06 (3H, *s*)	55.3	C_9	$3H_{10\text{-OMe}}$	3.81 (3H, *s*)	55.7
OCH_2O	6.34 (2H, *s*)	101.4	C_1, C_2	$2H_{OCH2O}$	6.14 (2H, *s*)	102.7

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Zhou *et al*., 2000).

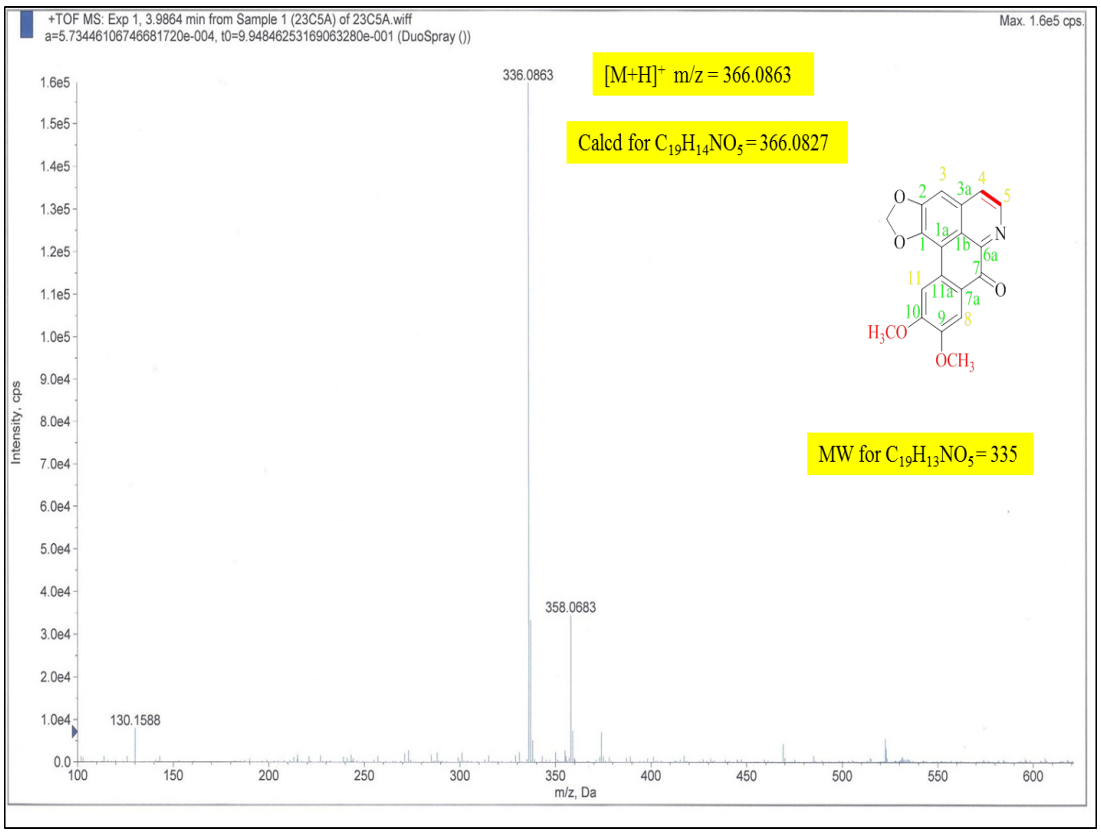


Figure 4.32: LCMS Triple TOF-MS spectrum of dicentrinone (**58**)

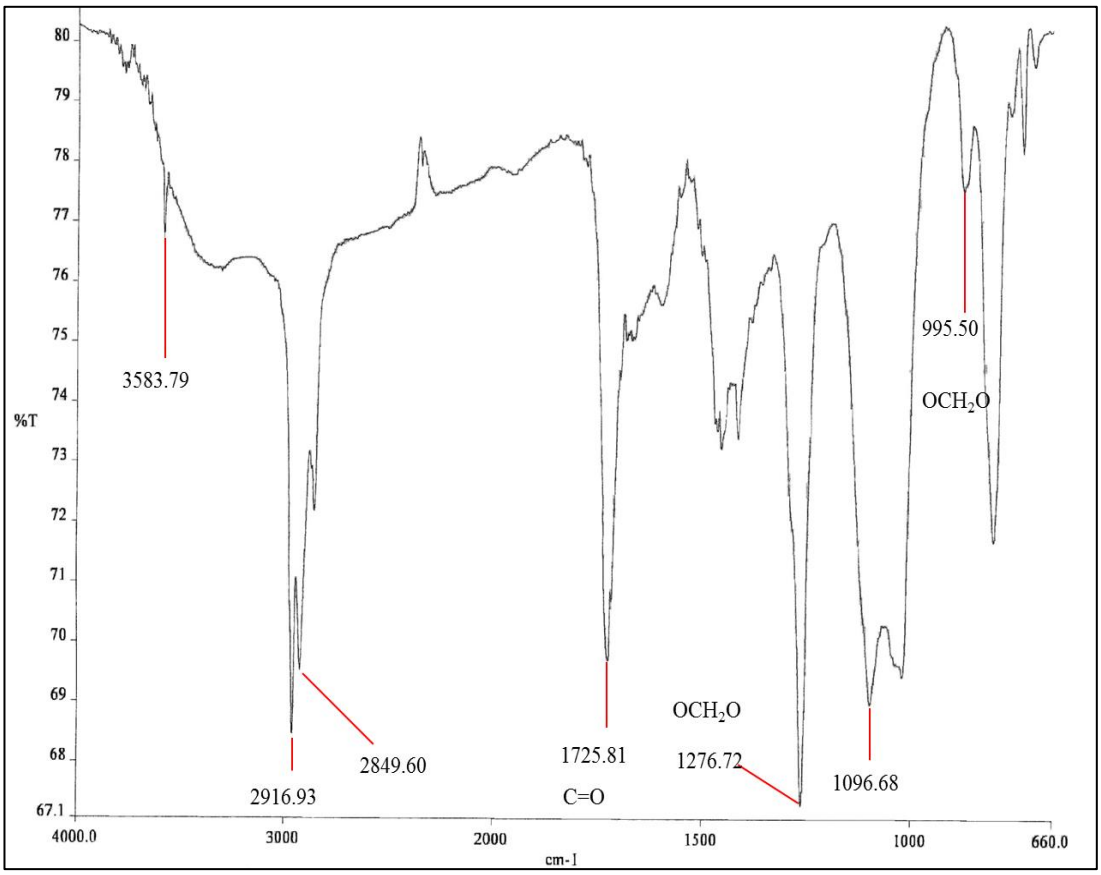


Figure 4.33: IR spectrum of dicentrinone (**58**)

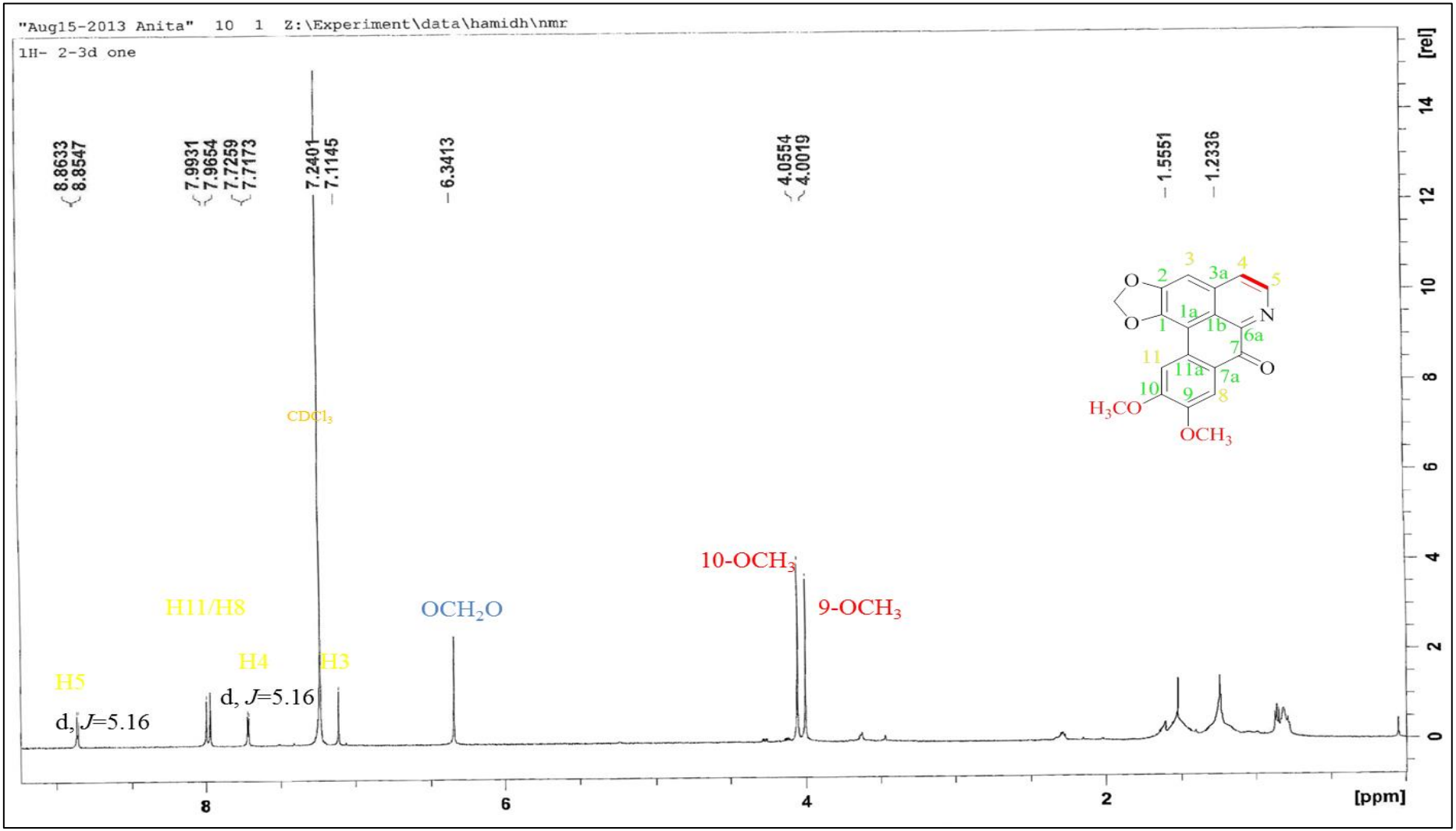


Figure 4.34: ^{1}H NMR spectrum of dicentrinone (**58**)

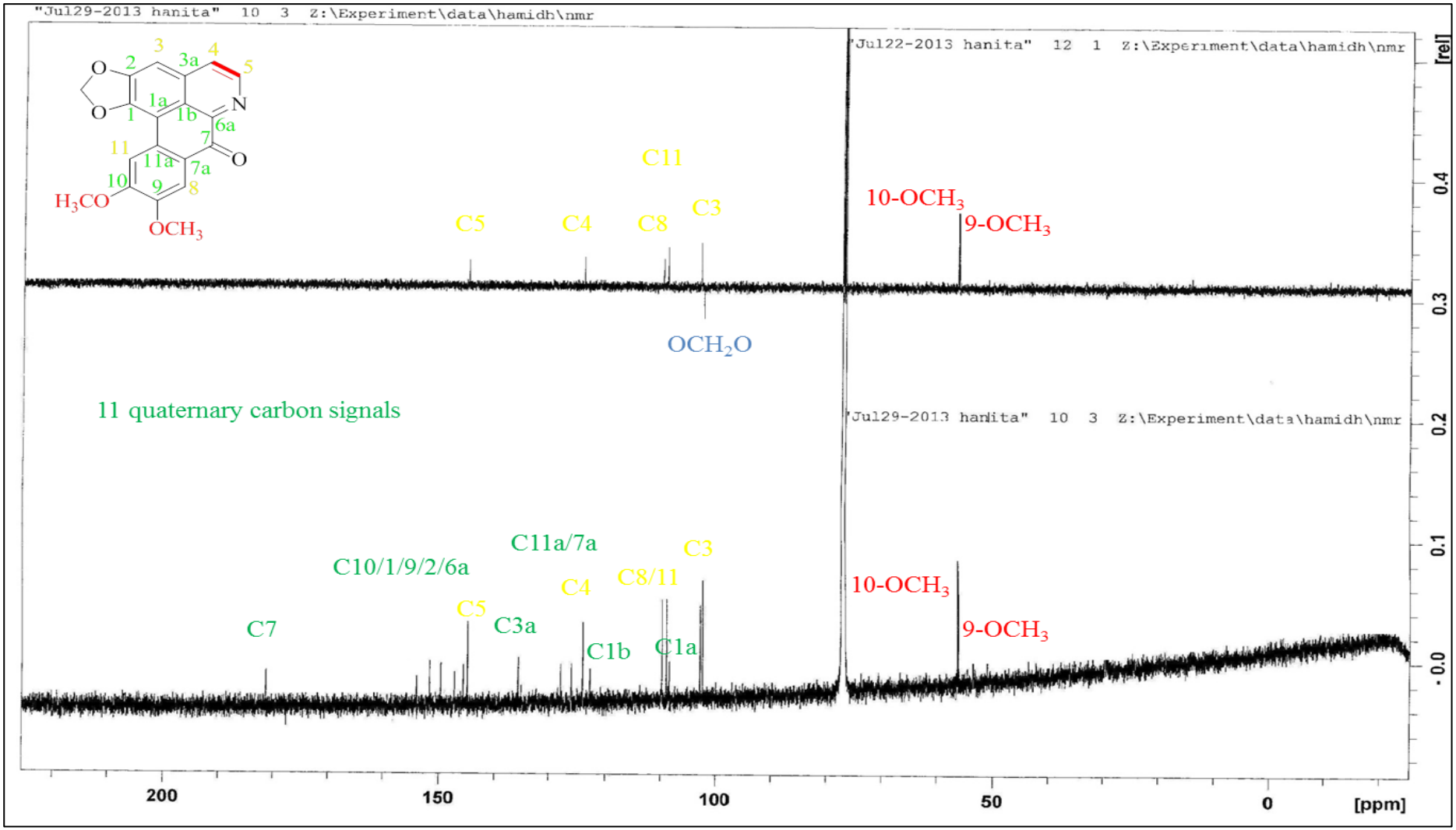


Figure 4.35: ^{13}C and DEPT 135- NMR spectrum of dicentrinone (**58**)

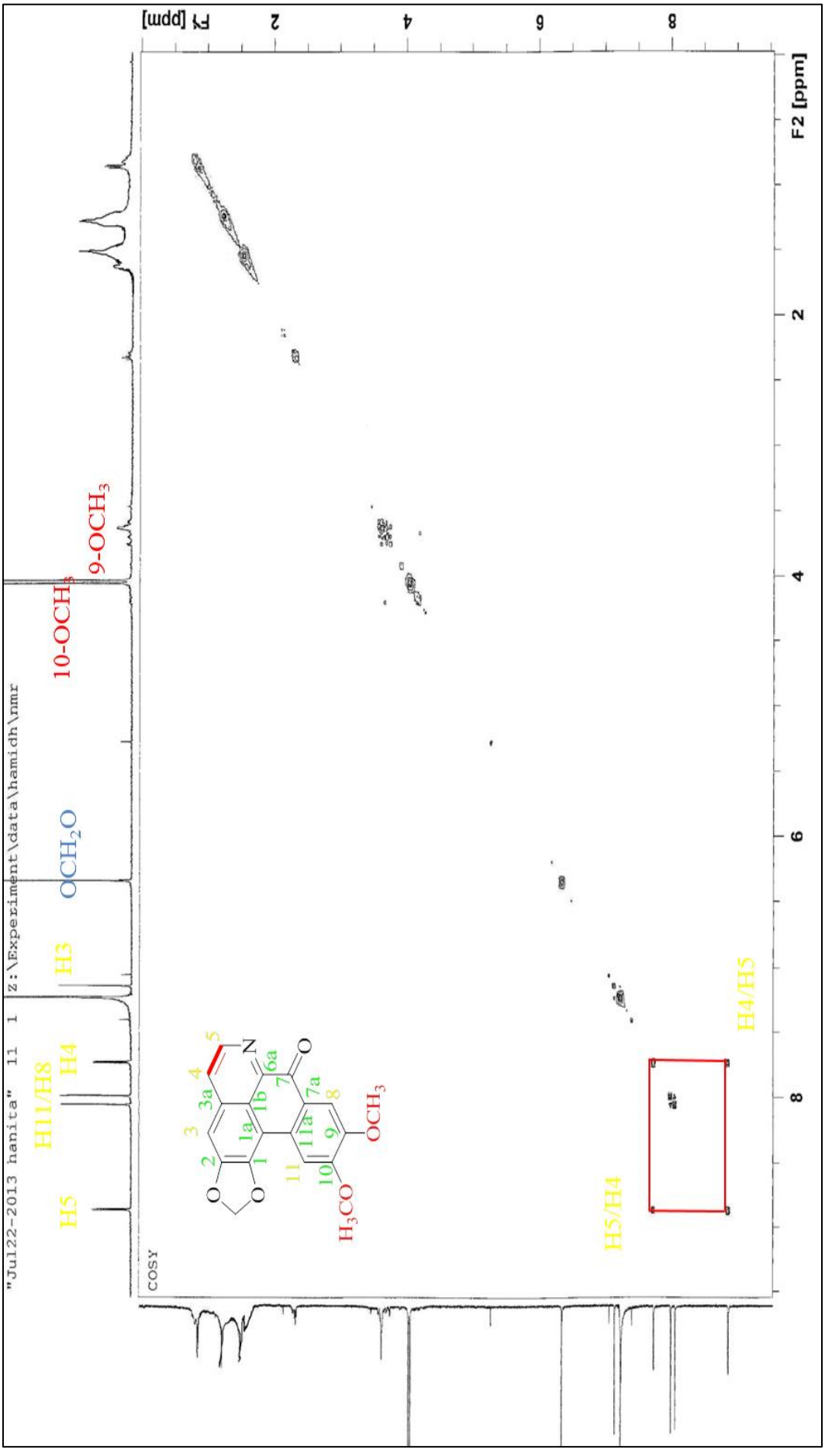


Figure 4.36: ^{1}H- ^{1}H COSY- NMR spectrum of dicentrinone (**58**)

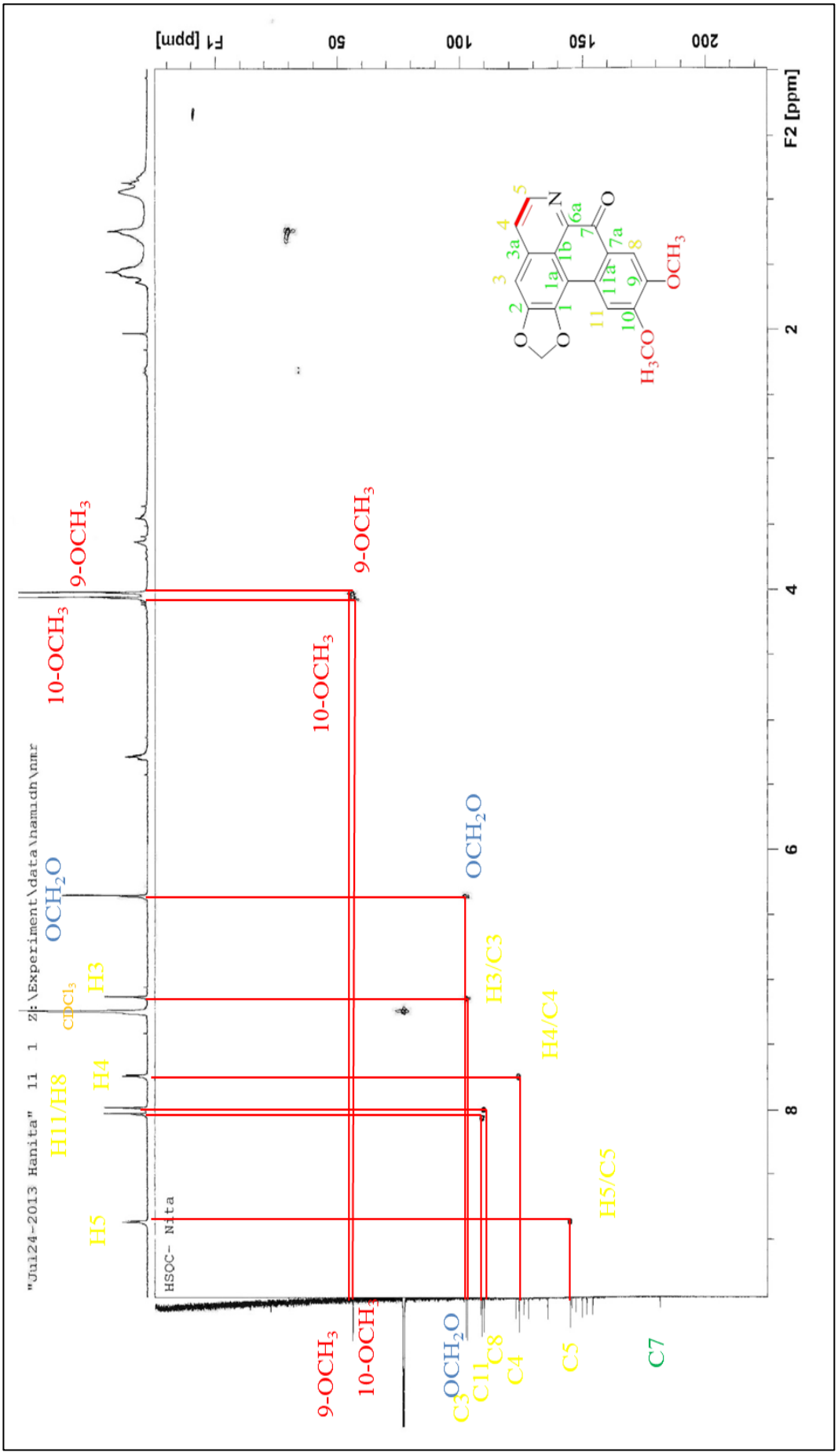


Figure 4.37: ^{1}H- ^{13}C HSQC- NMR spectrum of dicentrinone (**58**)

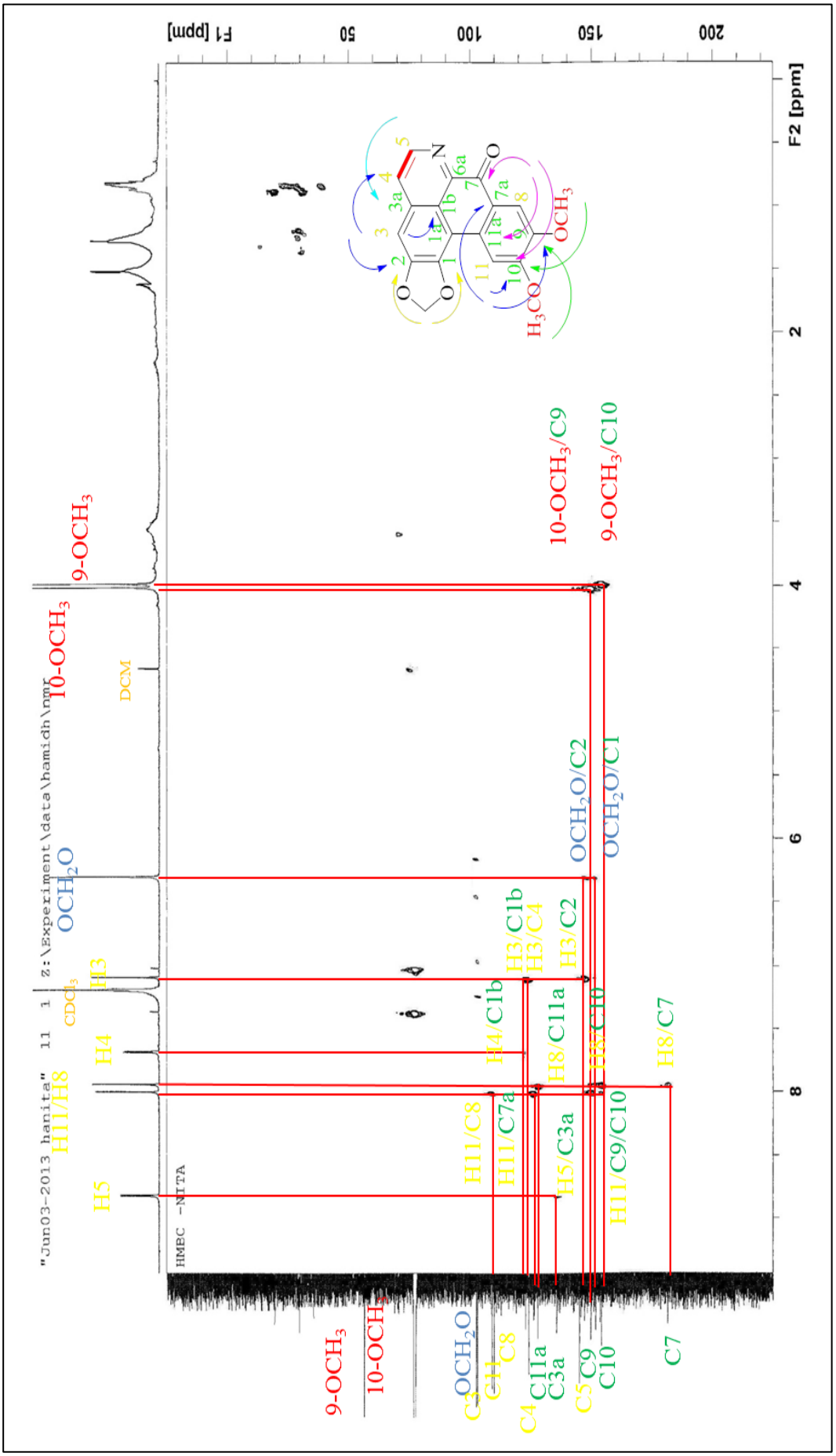


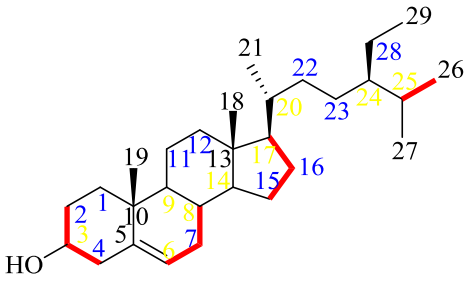
Figure 4.38: ^{1}H- ^{13}C HMBC- NMR spectrum of dicentrinone (**58**)

4.3 Compounds isolated from the stem bark of *Phoebe grandis* (KL 4994)

The isolation of phytochemicals from the stem bark of *Phoebe grandis* (KL 4994) afforded two known compounds from the hexane extract which were β-sitosterol (**59**) and stigmasterol (**60**).

Further investigation on the dichloromethane extract of the stem bark of *Phoebe grandis* (KL 4994) species led to the isolation of five known aporphine alkaloids; namely boldine (**5**), N-methyllaurotetanine (**51**), reticuline (**61**) and laurolitsine (**6**) or norboldine.

4.3.1 β-sitosterol (59)



59

Compound **59** with $[\alpha]^{25}_{D}$ = -36.6˚ (c= 0.15, $CHCl_3$) was isolated from hexane extract and methanol extract of the plant. From the positive tests for steroids and alcohols, it is assumed to be a compound containing steroidal nucleus. It is white crystalline needles like substance with melting point 137˚C which was in good agreement with the melting point given for β-sitosterol in the literature [(Hill *et al*., 1991), m.p 136-137˚C]. The UV max value of **59** is 251 nm, respectively. The mass spectrum LCMS-Q TOF (Figure 4.39) showed a molecular ion $[M+H]^+$ peak at m/z 415.7750 which corresponds to the molecular formula $C_{29}H_{50}O$ (calcd for $C_{29}H_{51}O$, 415.7180).

The IR spectrum (Figure 4.40) showed an intensely broad band at 3432 cm^{-1} was observed for the O-H bond vibrations of hydroxyl group. The corresponding C=C vibrations was shown around 1637 cm^{-1} as weakly intense band. The stretching and bending vibrations of methyl part were noticed by the intense band 2936 cm^{-1} and medium intensity band at 1460 cm^{-1}.

The ^{1}H-NMR spectra (Fig. 4.41) of **59**, was seen that H-3 proton appeared at δ 3.50 as a triplet of a double doublet with a *J* value of 4.5, 4.2, 3.8 Hz and H-6 olefinic proton showed a multiplet at δ 5.33. Moreover, six methyl proton signals appeared as three methyl singlets at δ 0.66, δ 0.81, δ 0.99; two methyl doublets that appeared at δ 0.90, δ 0.79; and a methyl triplet at δ 0.82. The ^{1}H NMR spectra data showed a proton corresponding to the proton connected to the C-3 hydroxy group which appeared as a triplet of doublet of doublets at δ 3.50. These assignments are in good agreement for the structure of β-sitosterol. In addition, the COSY correlations (Figure 4.44) between H-2 and H-3, H-3 and H-4, H-6 and H-7, H-7 and H-8, H-15 and H-16, H-16 and H-17, and H-25 and H-26 signals and the NOESY correlations (Figure 4.47) between H-6 and H-4, H-3 and H-1 signals also supported the structure of **59**.

The ^{13}C-NMR (Figure 4.42) has shown recognisable signals 140.7 and 121.7 ppm, which are assigned C5 and C6 double bonds respectively as in Δ^{5} spirostene (Agrawal *et al*., 1985). The value at 19.8 ppm corresponds to angular carbon atom (C19). Extensive interpretation of DEPT spectra (Figure 4.43), COSY (Figure 4.44), HSQC (Figure 4.45 and Figure 4.45.1), and HMBC (Figure 4.46 and Figure 4.46.1) experiments of **59**, let us established all connectivities, with the presence of twenty nine carbon signals including six methyl, eleven methylenes, nine methine and three quaternary carbons, to accomplish the full assignment of all ^{1}H and ^{13}C NMR signals

(Table 4.7). Thus, the structure of **59** was assigned as β-sitosterol that was also consistent to the reported literature values (Habib *et al*., 2007; Jamal *et al*., 2009). The ^{13}C-NMR data of **59** were also quite similar with the data in the literature of β-sitosterol, respectively (Chaturvedula & Prakash, 2012; Conolly & Hill, 1994).

Table 4.7: ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz) and 2D (HMBC and HSQC) NMR data of β-sitosterol (**59**) and the literature data.

	59 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	1.84, 1.05	37.3	C_3, C_5.	$2H_1$		37.5
2	1.82, 1.81	31.7	C_3, C_5.	$2H_2$		31.9
3	3.50 (*tdd*, 1H, *J*=4.5 4.2, 3.8)	71.8	-	H_3	3.53 (*tdd*,1H, *J*=4.5, 4.2, 3.8)	72.0
4	2.27, 2.22	42.3	C_2, C_{10}, C_3, C_6, C_5.	$2H_4$		42.5
5		140.7	-	-		140.9
6	5.33 (1H, *m*)	121.7	C_8, C_{10}, C_9.	H_6	5.36 (t, 1H, *J*=6.4)	121.9
7	1.97, 1.94	31.9	C_9, C_{14}.	$2H_2$		32.1
8	1.46	31.9	-	H_8		32.1
9	0.87	50.2	-	H_9		50.3
10		36.2	-	-		36.7
11	1.48, 1.47	21.1	-	$2H_{11}$		21.3
12	2.00, 1.13	39.8	-	$2H_{12}$		39.9
13		42.3	-	-		42.6
14	0.97	56.8	-	H_{14}		56.9
15	1.55, 1.04	24.3	C_{13}	$2H_{15}$		26.3
16	1.82, 1.24	28.2	C_{21}, C_{13}.	$2H_{16}$		28.5
17	1.08	56.1	C_{22}	H_{17}		56.3
18	0.66 (*s*, 3H)	11.9	C_{12}, C_{13q}, C_{17}, C_{14}.	$3H_{18}$	1.01 (*s*, 3H)	12.0
19	0.81 (*s*, 3H)	19.8	-	$3H_{19}$	0.68 (*s*, 3H)	19.0
20	1.33	36.5	-	H_{20}		36.3
21	0.90 (*d*, 3H, *J*=6.5)	18.8	C_{20}, C_{22}, C_{17}.	$3H_{21}$	0.93 (*d*, 3H, *J*=6.5)	19.2
22	1.29, 0.98	34.0	-	$2H_{22}$		34.2
23	1.14, 1.14	26.1	-	$2H_{23}$		26.3
24	0.91	45.9	-	H_{24}		46.1
25	1.63	29.2	C_{28}, C_{24}.	H_{25}		29.4
26	0.79 (*d*, 3H, *J*=6.8)	19.0	C_{25}, C_{24}, C_{28}.	$3H_{26}$	0.83 (*d*, 3H, *J*=6.4)	20.1
27	0.99 (*s*, 3H)	19.4	C_{20}, C_9, C_5.	$3H_{27}$	0.81 (*d*, 3H, *J*=6.4)	19.6
28	1.25, 1.20	23.1	C_{29}, C_{23}, C_{25}, C_{24}.	$2H_{28}$		23.3
29	0.82 (*t*, 3H, *J*=7.4)	12.0	C_{26}, C_{28}, C_{25}, C_{24}.	$3H_{29}$	0.84 (*t*, 3H, *J*=7.2)	12.2

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Chaturvedula & Prakash, 2012).

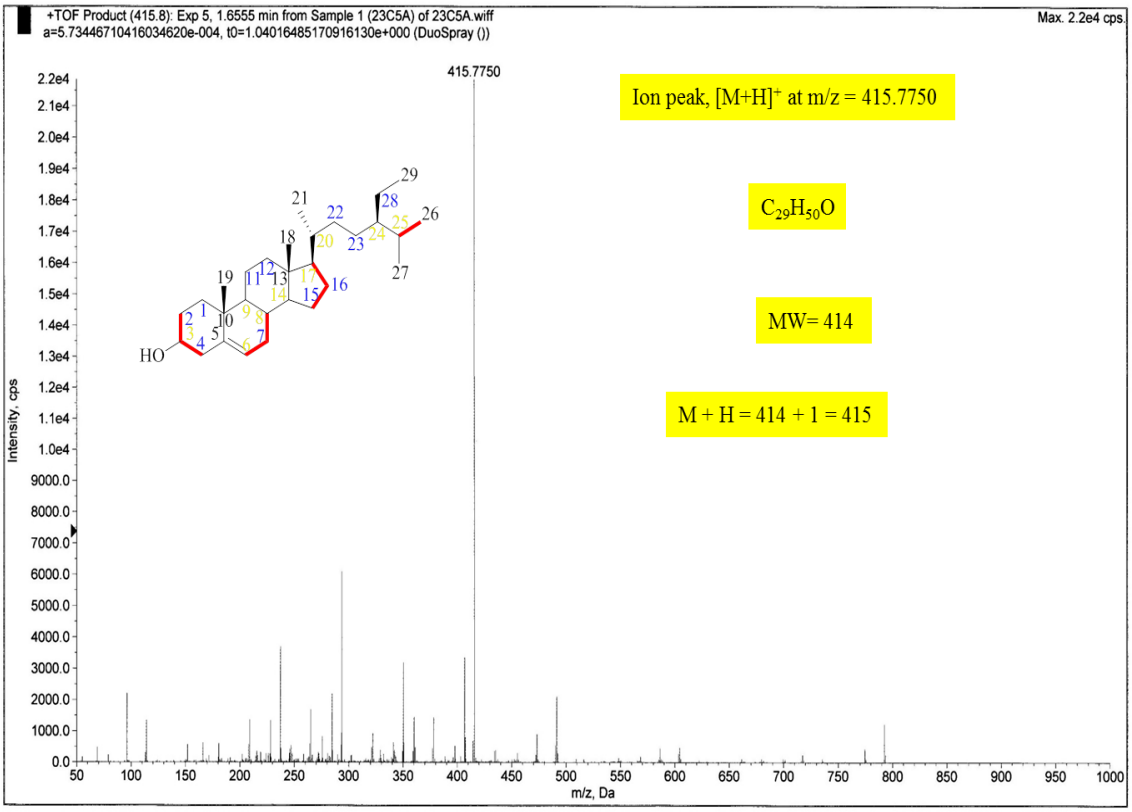


Figure 4.39: LCMS- Q TOF spectrum of β-sitosterol (**59**).

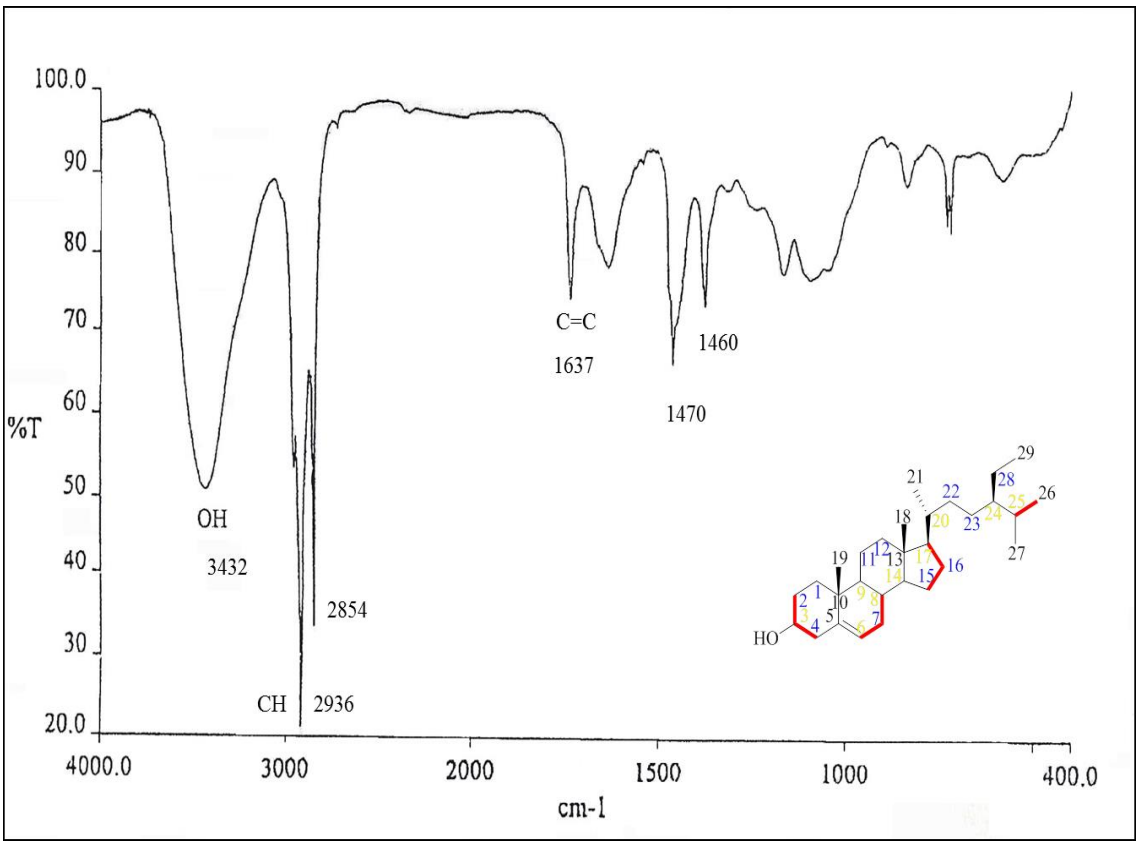


Figure 4.40: IR spectrum of β-sitosterol (**59**)

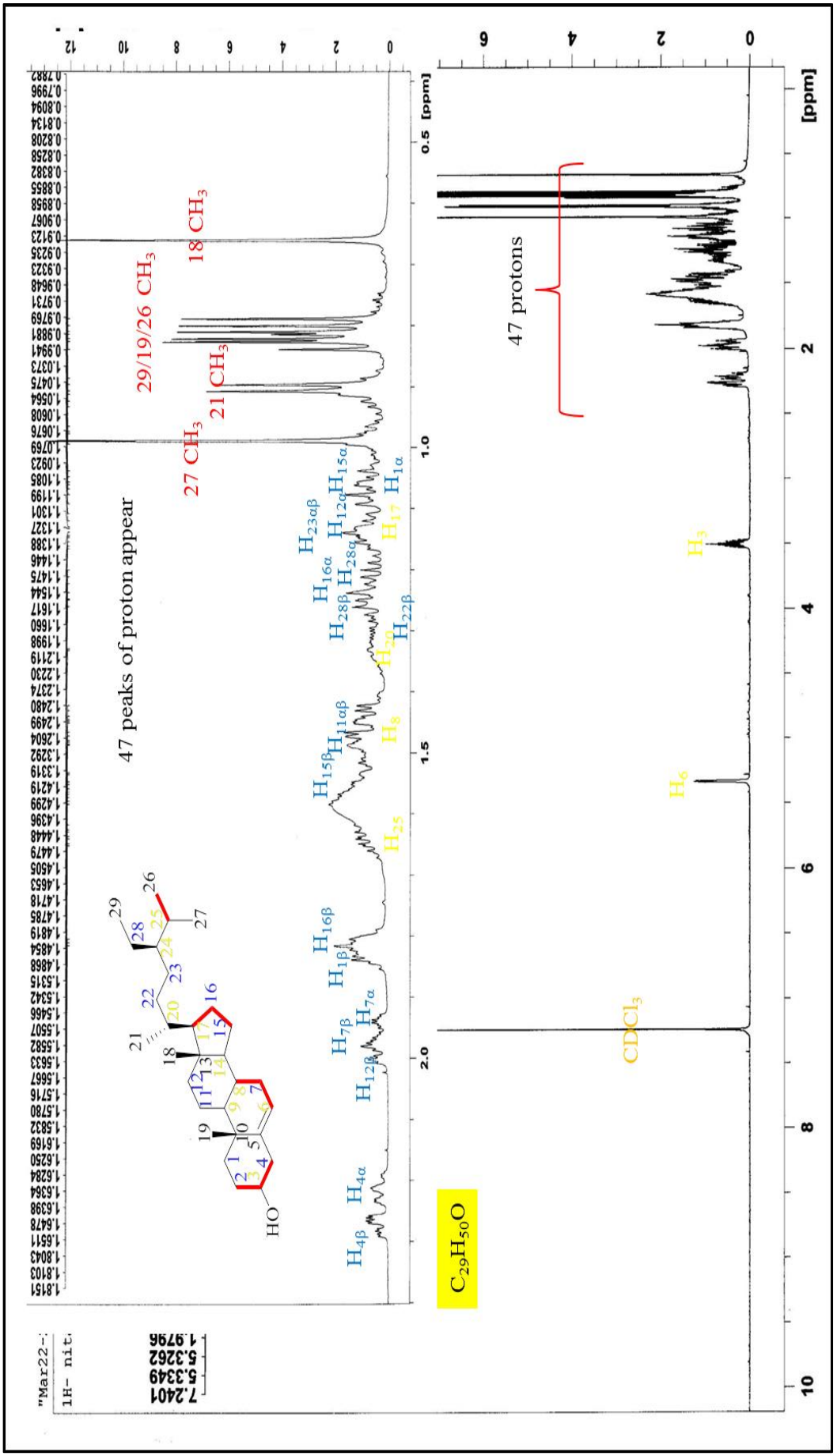


Figure: 4. 41: ^{1}H NMR spectrum of β-sitosterol (**59**).

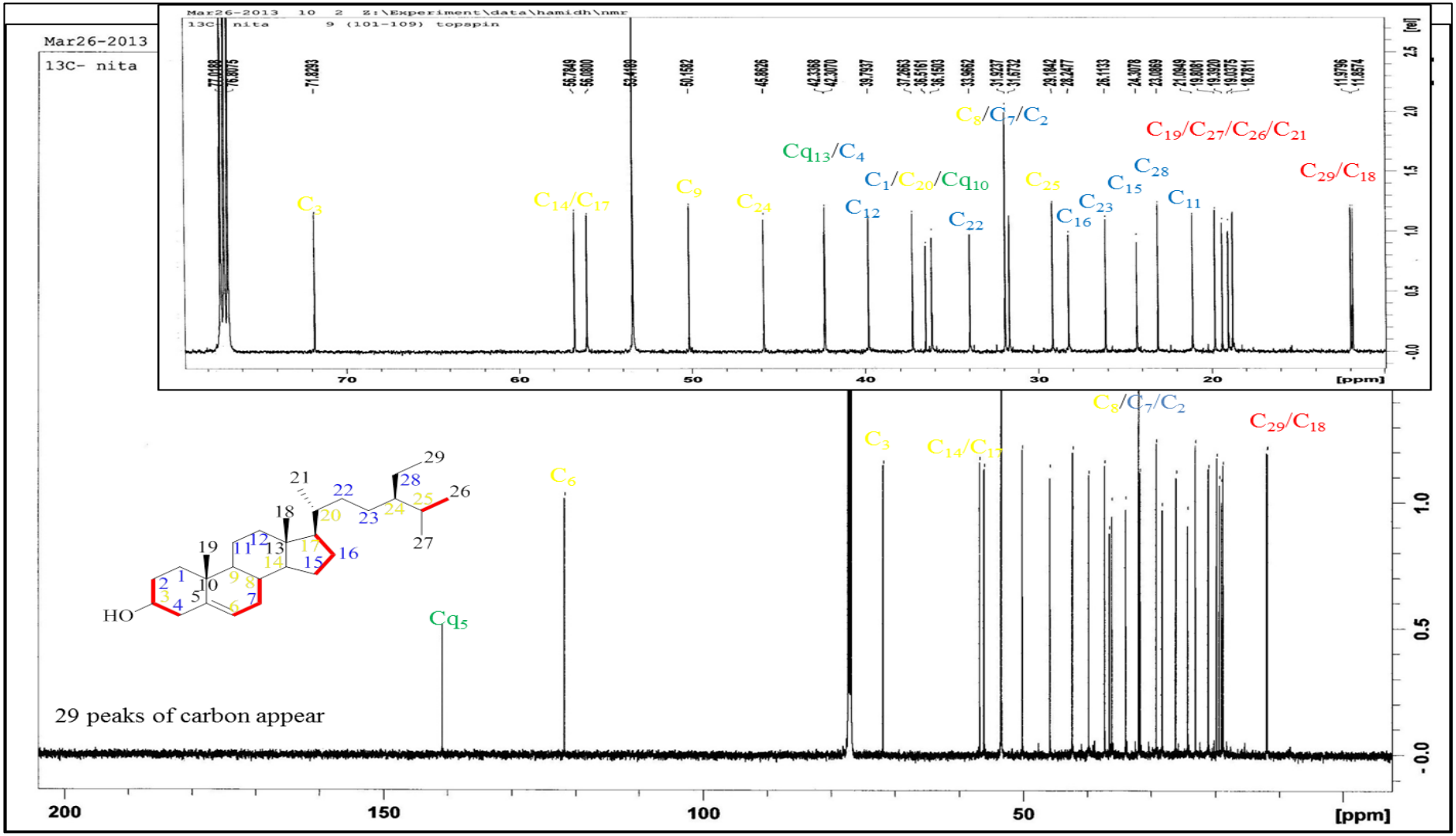


Figure 4.42: ^{13}C NMR spectrum of β-sitosterol (**59**).

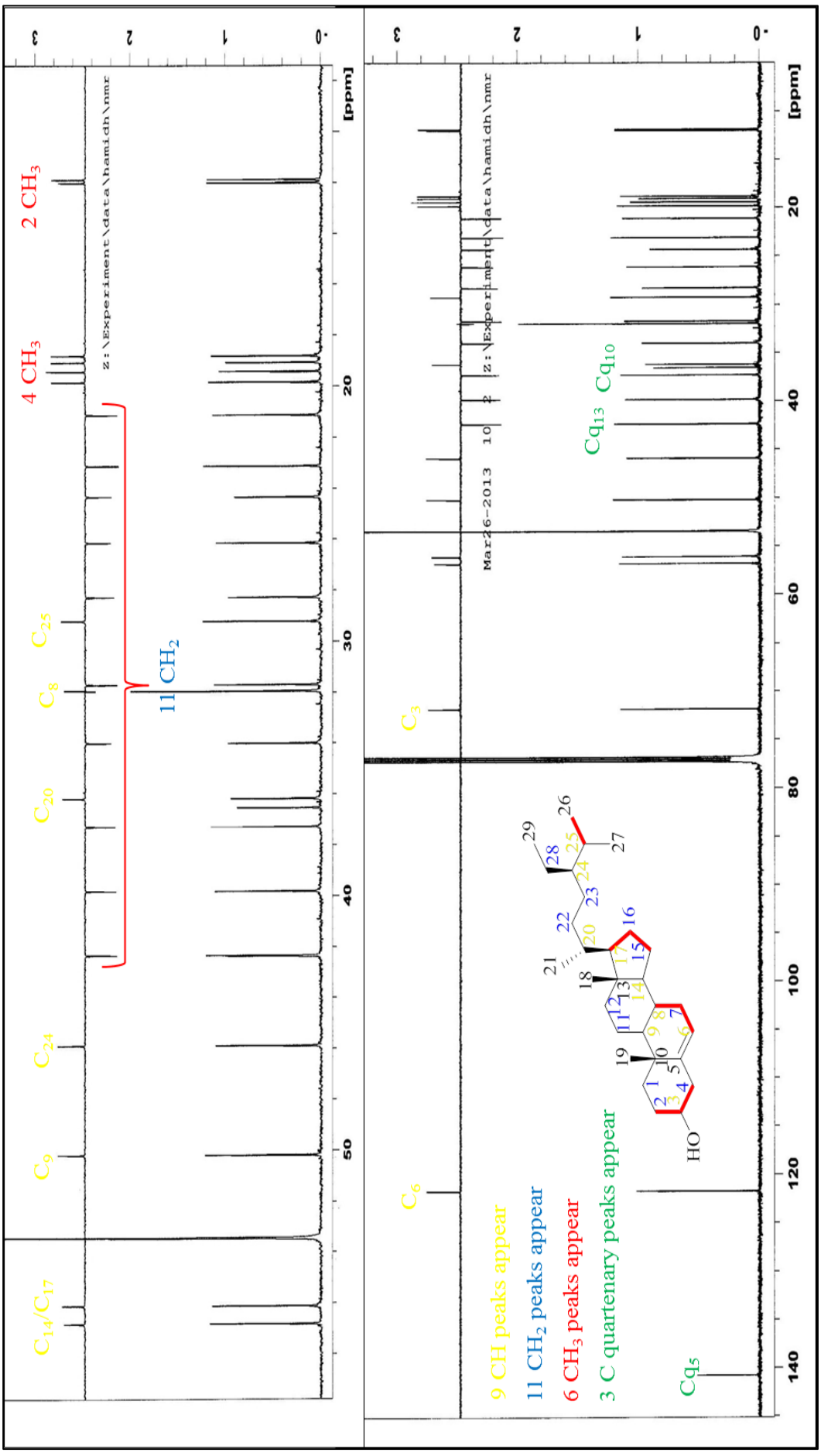


Figure 4.43: DEPT spectrum of β-sitosterol (**59**)

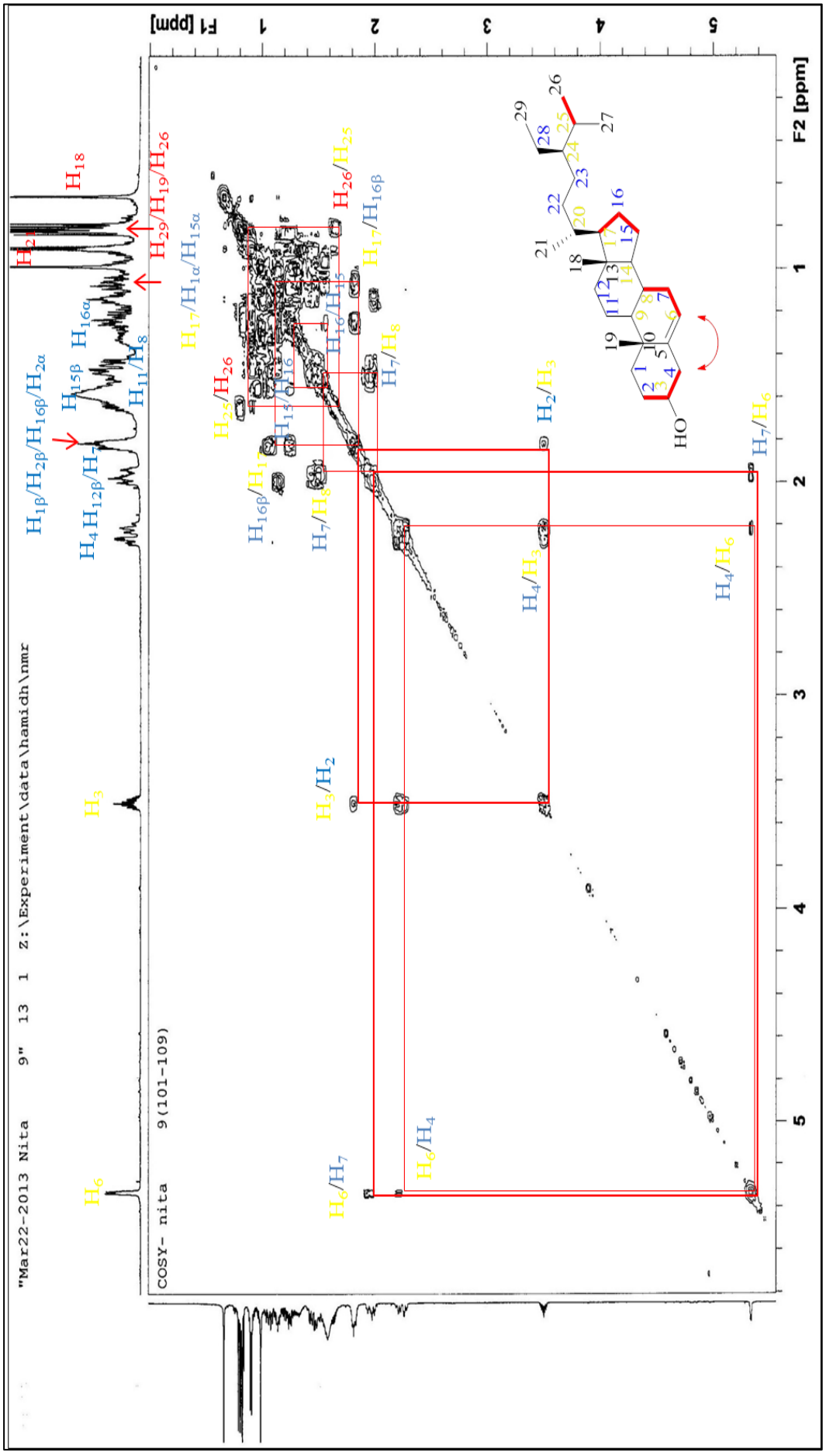


Figure 4.44: COSY spectrum of β-sitosterol (**59**).

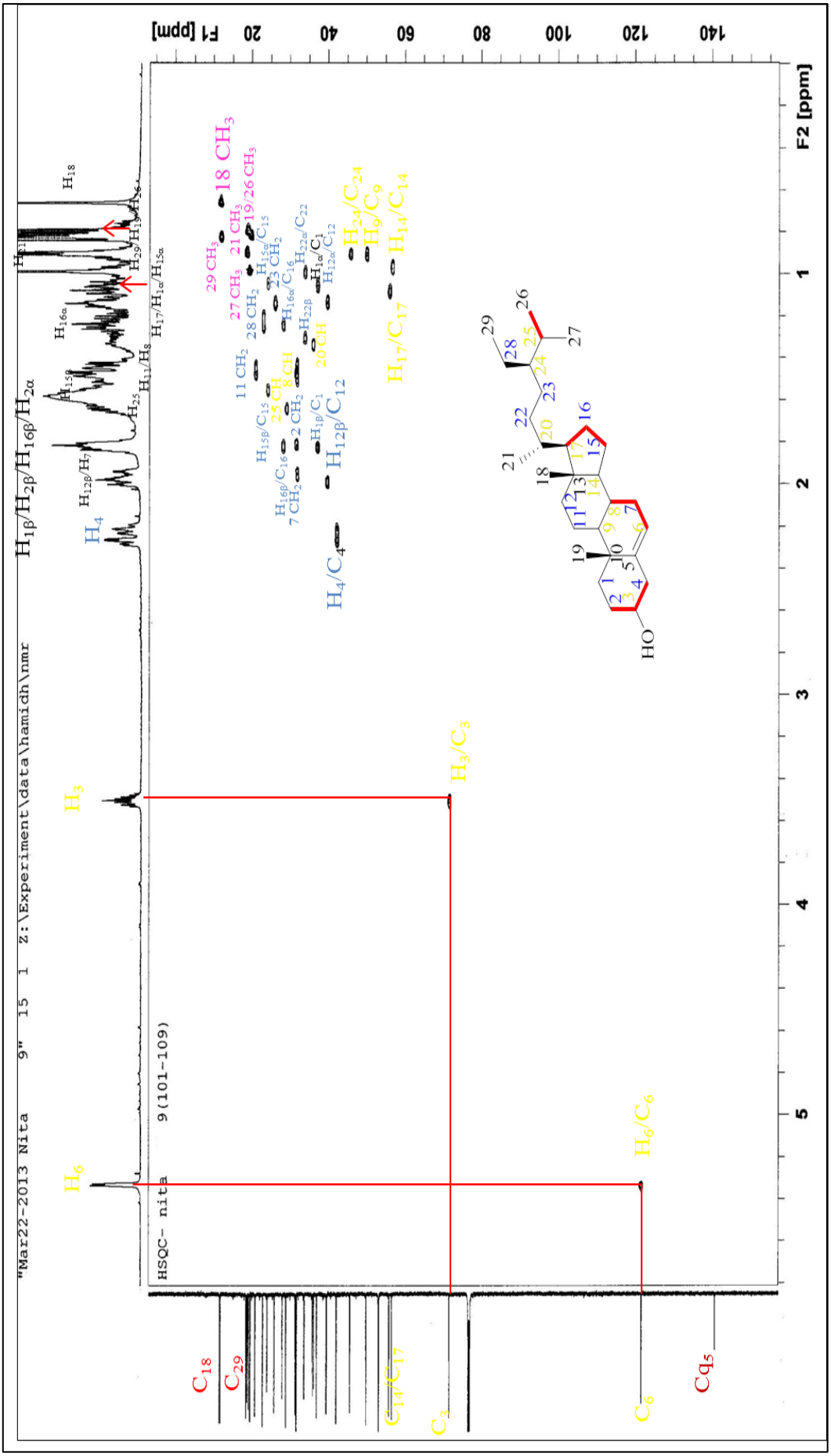


Figure 4.45: HSQC spectrum of β-sitosterol (**59**).

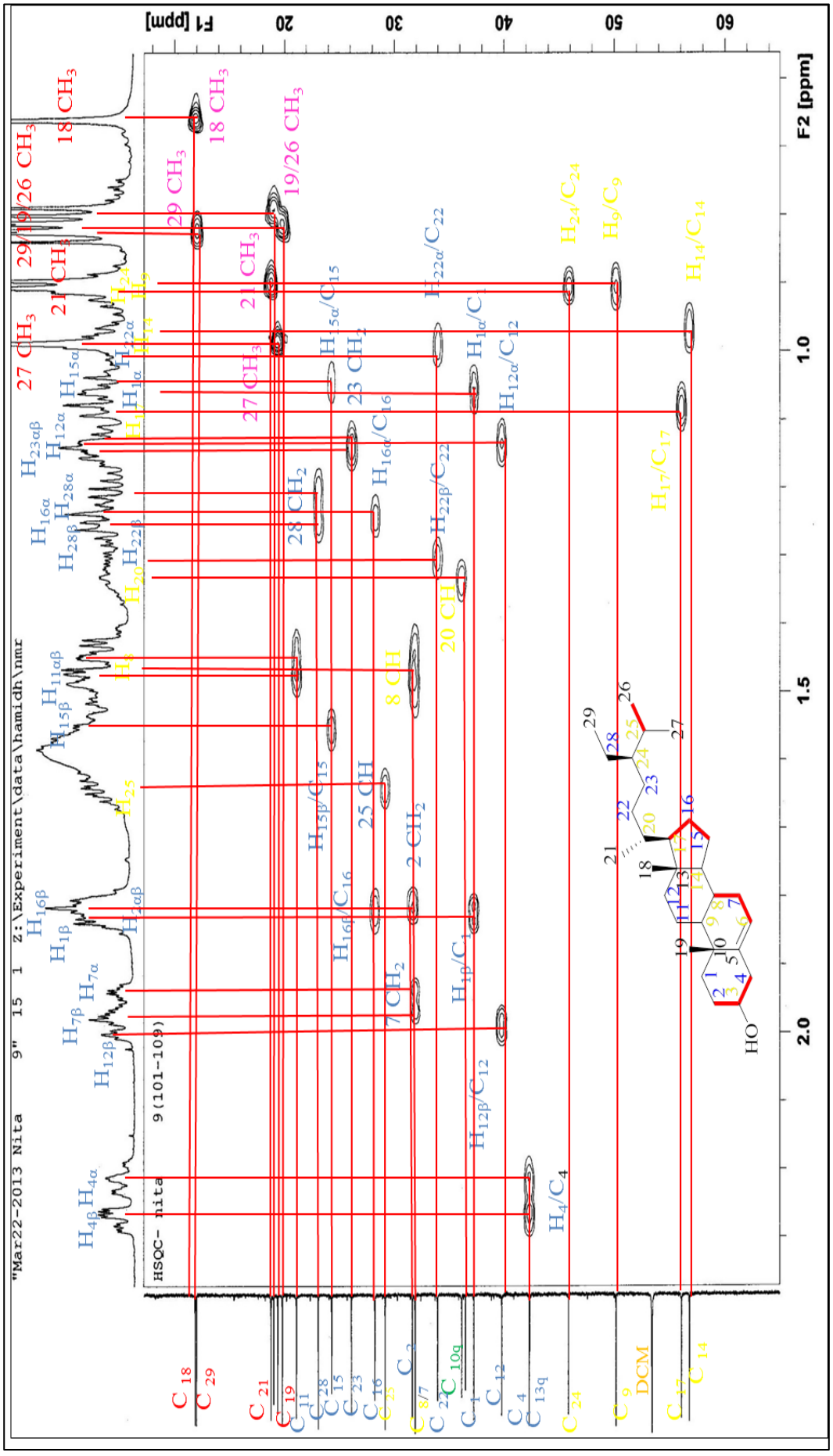


Figure 4.45.1: An Expanded of HSQC spectrum of β-sitosterol (**59**).

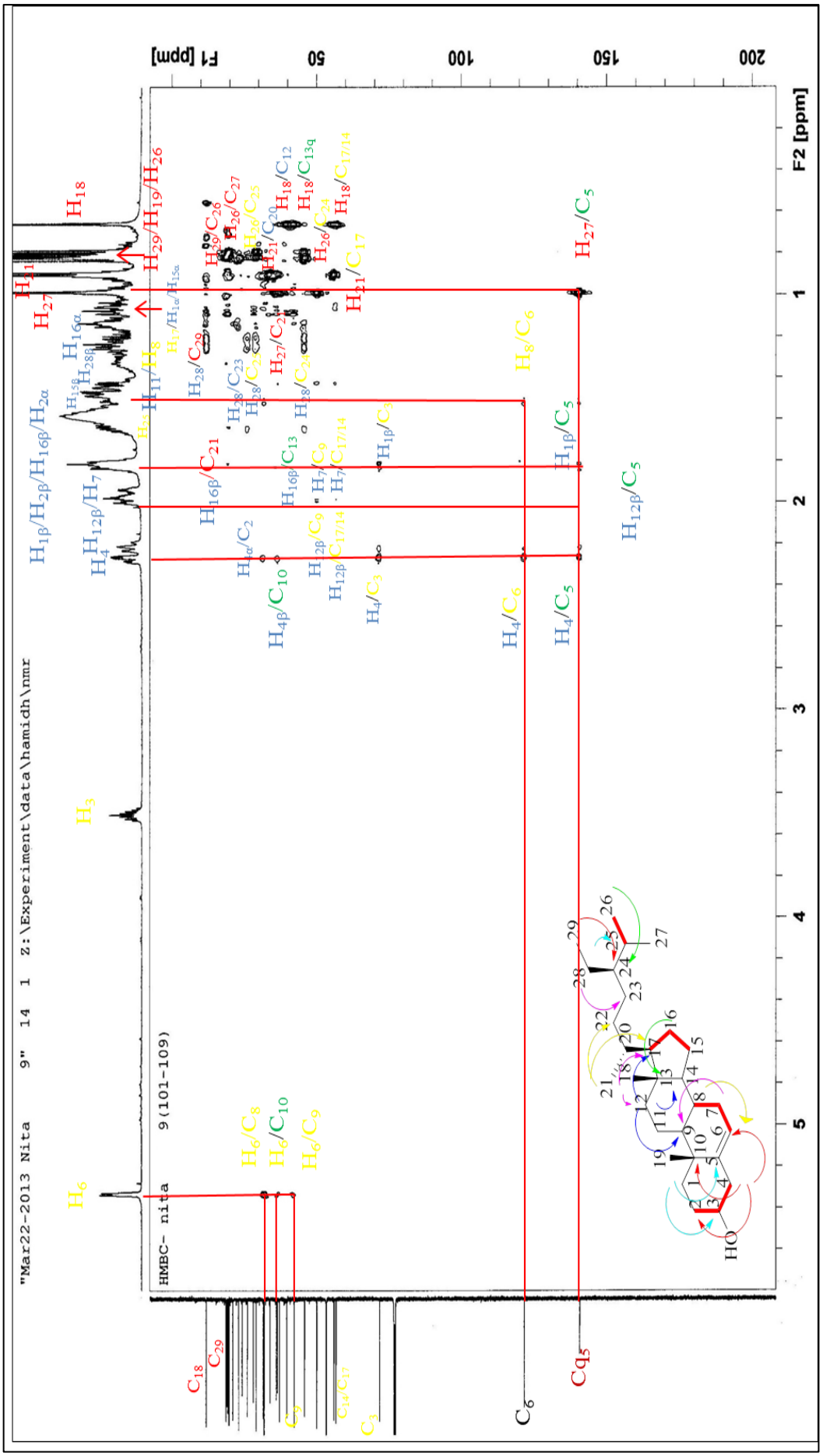


Figure 4.46: HMBC spectrum of β-sitosterol (**59**).

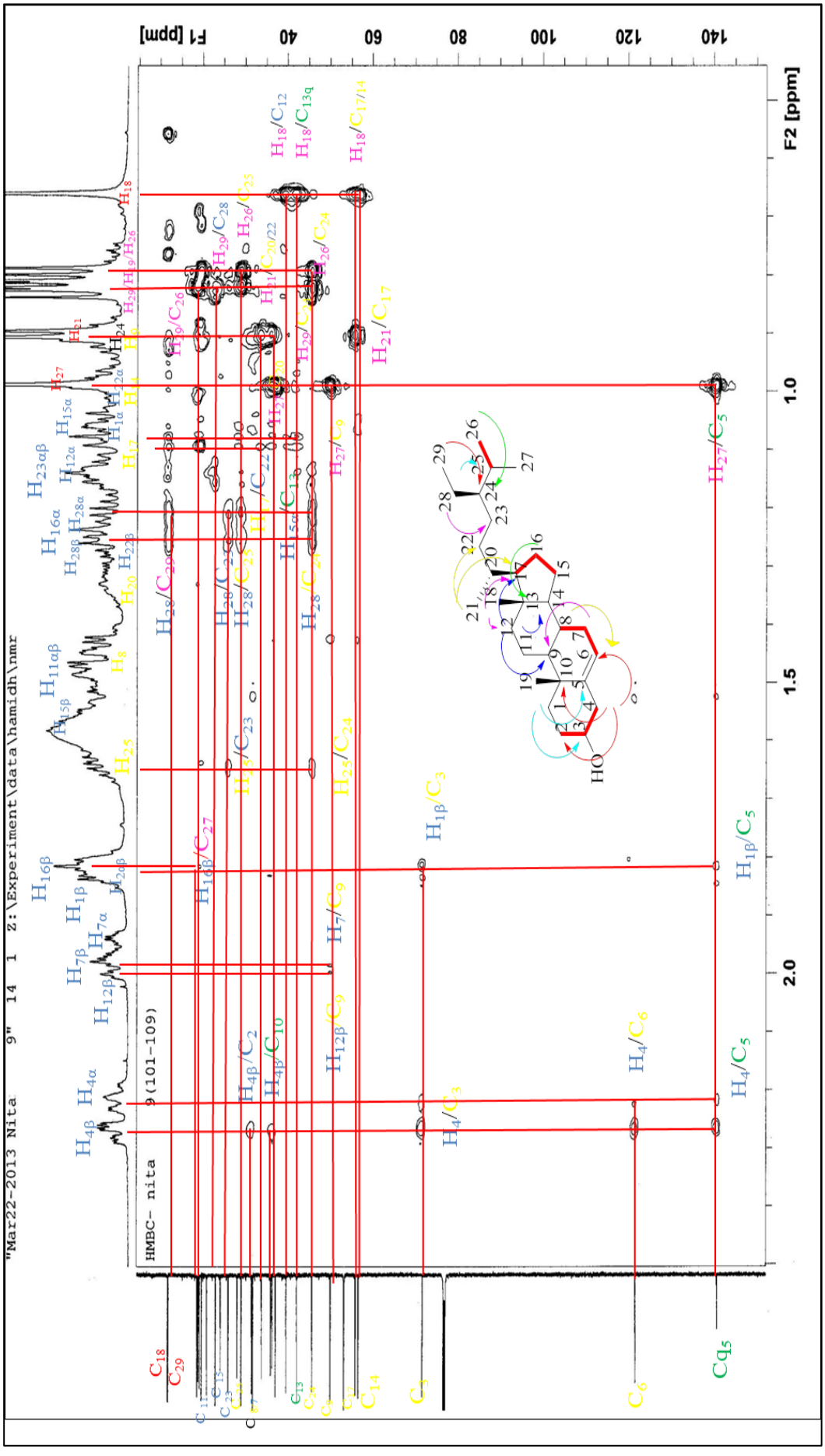


Figure 4.46.1: An Expanded of HMBC spectrum of β-sitosterol (**59**).

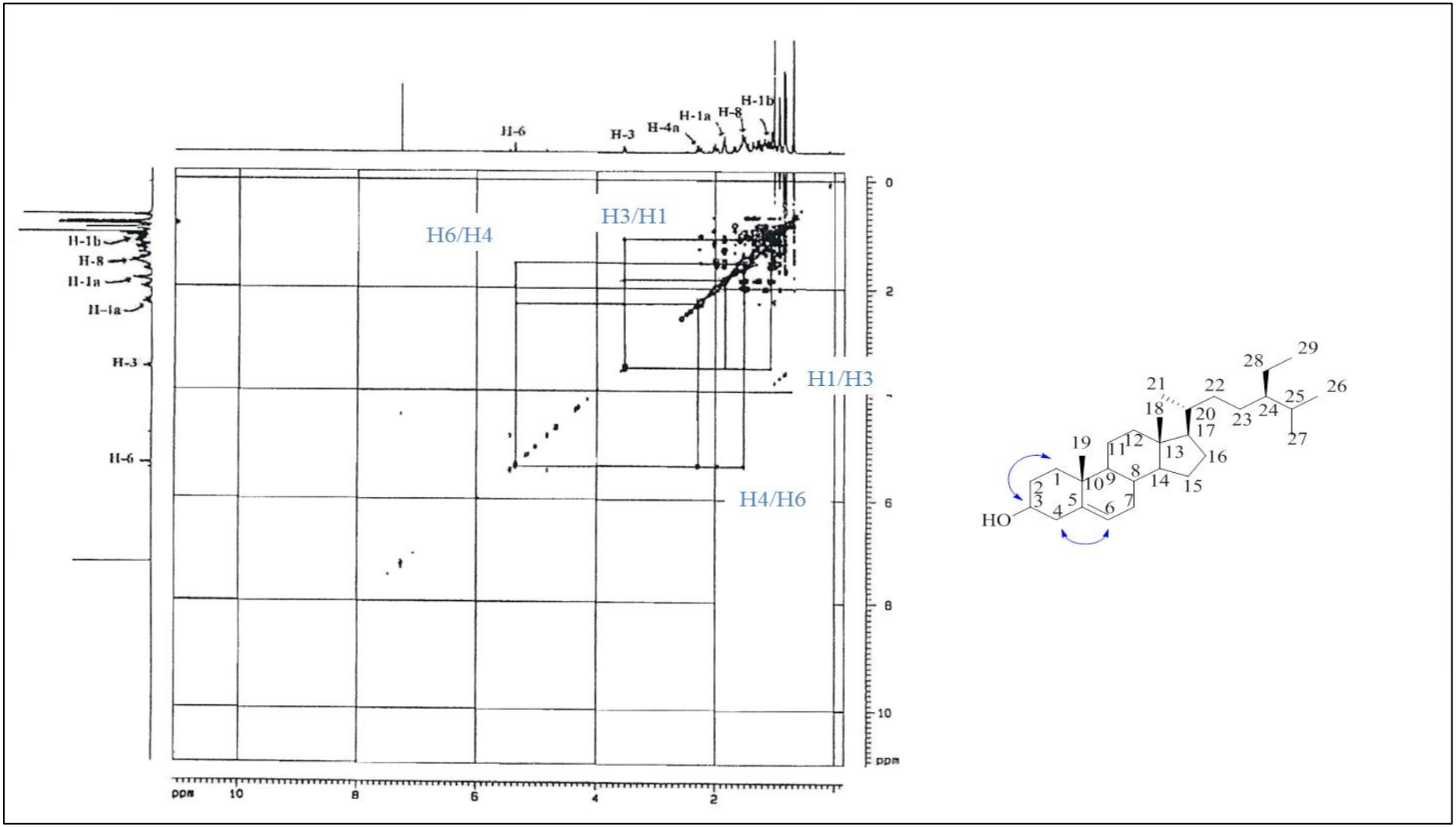
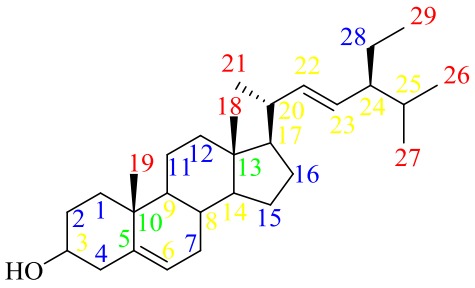


Figure 4.47: NOESY spectrum of β-sitosterol (**59**).

4.3.2 Stigmasterol (60)



60

Compound **60** was isolated from the hexane extract of the barks of *Phoebe grandis*. This compound was obtained as white powder m.p 158-160°C [(Schwartz & Wall, 1955), 166-168°C)]. The UV max value of **60** is 257 nm, respectively. The EIMS spectrum (Figure 4.48) showed the molecular ion peak at m/z 412 consistent with the molecular formula $C_{29}H_{48}O$, which was supported by the ^{13}C NMR spectral data.

The IR spectrum (Figure 4.49) showed a broad strong band at 3351 cm^{-1} was due to the presence of a hydroxyl group. The absorption band at 1656 cm^{-1} was typical of C=C double bond. The absorption band observed at 2934 cm^{-1} and 1459 cm^{-1} were due to the stretching of C-H bonding from methyl and methylene groups.

The mass fragmentation patterns (Figure 4.50) showed that the molecular ion of **60** was observed at m/z 412. The loss of water from the molecular ion was indicated by the presence of a fragment ion at m/z 394 in the mass spectrum. This implied the existence of a hydroxyl group at C-3. The subsequent fragment at m/z 379 was due to the loss of a methyl group. The characteristic feature of the fragmentation of **60** is the presence of a fragment ion peak at m/z 271 due to the loss of the side chain followed by the loss of two hydrogen atoms. Based of the above data, this compound **60** can be identified as stigmasterol.

The deduction for the determination of structure of **60** was supported by ^{1}H-NMR analysis (Figure 4.51). The presence of an olefinic proton was indicated by a multiplet signal at δ 5.36 attached to C-6. A doublet of doublets at δ 5.14 (*J*= 15.2, 8.5 Hz) and another doublet of doublets δ 5.02 (*J*= 15.2, 8.5 Hz) were due to the sp^2 methine protons H-22 and H-23 of the side chain, respectively. The large coupling constant value of 15.2 Hz showed that the two protons have a *trans* configuration. ^{1}H-NMR spectra of **60** showed the presence of two methyl singlets at δ 0.66 and 0.99; three methyl doublets that appeared at δ 0.79, 0.81 and 0.90; and a methyl triplet at δ 0.82. Liebermann-Burchard reaction indicated compound **60** is having a sterol skeleton (Kandati *et al*., 2012; (Hosahally *et al*., 2012). The proton corresponding to the H-3 of a sterol moiety was appeared as a triplet of doublet of doublets at δ 3.52.

^{13}C-NMR spectra data (Figure 4.52) showed the presence of 29 carbons and supported by the mass spectra (Figure. 4.48), suggested the molecular formula as $C_{29}H_{48}O$. The above spectral data supported the presence of sterol skeleton having a hydroxyl group at C-3 position, with two double bonds at C-5/C-6 and C-22/C23 with six methyl groups. The value at 19.4 ppm corresponds to angular carbon atom (C19). Thus, the structure of **60** was assigned as the known compound stigmasterol. Moreover, the physical and spectral data are consistent to the reported literature values (Habib *et al*., 2007; Jamal *et al*., 2009; Moghaddam *et al*., 2007; Chaturvedula & Prakash, 2012; Hussain et al., 2008) of stigmasterol and **60** was confirmed as stigmasterol.

Table 4.8: ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data of stigmasterol (**60**) and the literature data.

	60 in $CDCl_3$		* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	δ_H (600MHz) (ppm, *J* in Hz)	δ_C (150MHz) (ppm)
1	-	37.2	-	37.6
2	-	31.6	-	32.1
3	3.52 (*tdd*,1H, *J*=4.5, 4.2, 3.8)	71.8	3.51 (*tdd*,1H, *J*=4.5, 4.2, 3.8)	72.1
4	-	42.3	-	42.4
5	-	140.7	-	141.1
6	5.36 (*m*, 1H)	121.7	5.31 (*t*, 1H, *J*=6.1)	121.8
7	-	31.9	-	31.8
8	-	31.9	-	31.8
9	-	50.1	-	50.2
10	-	36.5	-	36.6
11	-	21.0	-	21.5
12	-	39.7	-	39.9
13	-	42.2	-	42.4
14	-	56.9	-	56.8
15	-	24.4	-	24.4
16	-	28.9	-	29.3
17	-	55.9	-	56.2
18	0.99 (*s*, 3H)	12.0	1.03 (*s*, 3H)	12.2
19	0.66 (*s*, 3H)	19.4	0.71 (*s*, 3H)	18.9
20	-	40.6	-	40.6
21	0.90 (*d*, 3H, *J*=6.5)	21.1	0.91 (*d*, 3H, *J*=6.2)	21.7
22	5.14 (*dd*, 1H, *J*= 15.2, 8.5)	138.1	5.14 (*m*, 1H)	138.7
23	5.02 (*dd*, 1H, *J*= 15.2, 8.5)	129.2	4.98 (*m*, 1H)	129.6
24	-	51.3	-	46.1
25	-	31.9	-	29.6
26	0.81 (*d*, 3H, *J*=6.8)	21.2	0.82 (*d*, 3H, *J*=6.6)	20.2
27	0.79 (*d*, 3H, *J*=6.6)	19.0	0.80 (*d*, 3H, *J*=6.6)	19.8
28	-	25.4	-	25.4
29	0.82 (*t*, 3H, *J*=7.4)	12.3	0.83 (*t*, 3H, *J*=7.1)	12.1

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

*(Chaturvedula & Prakash, 2012).

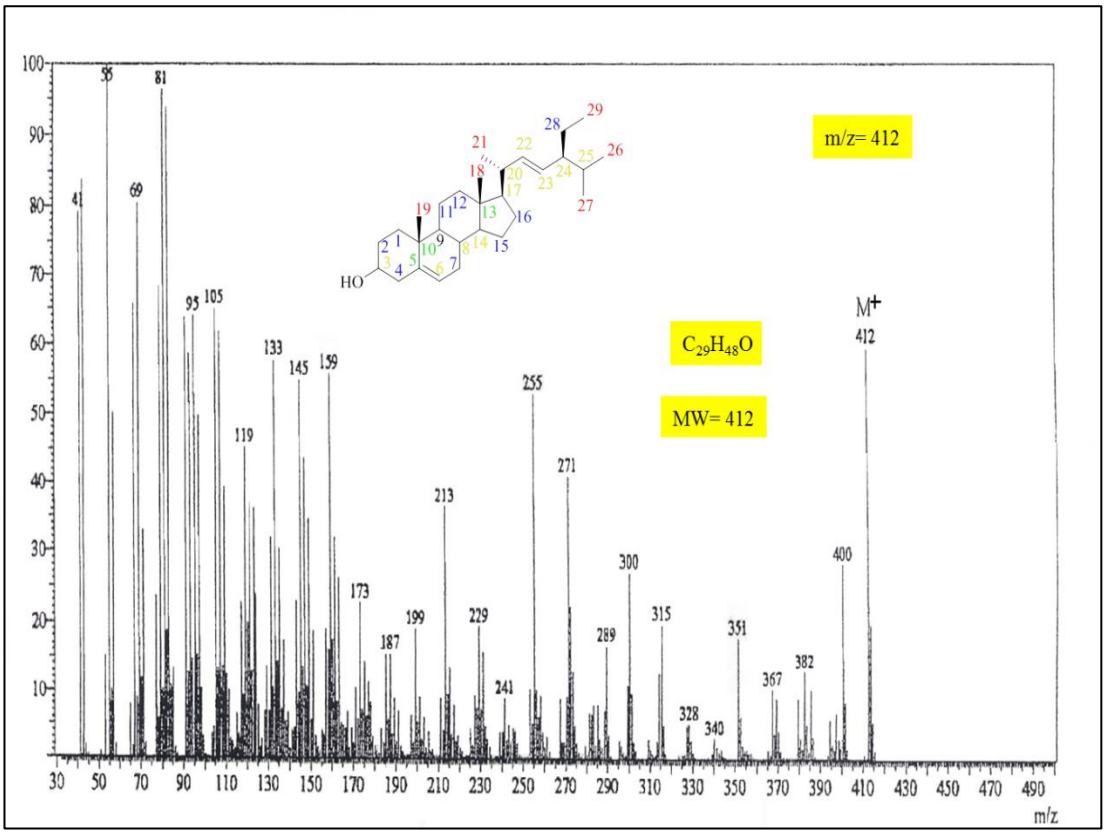


Figure 4.48: EIMS spectrum of stigmasterol (**60**).

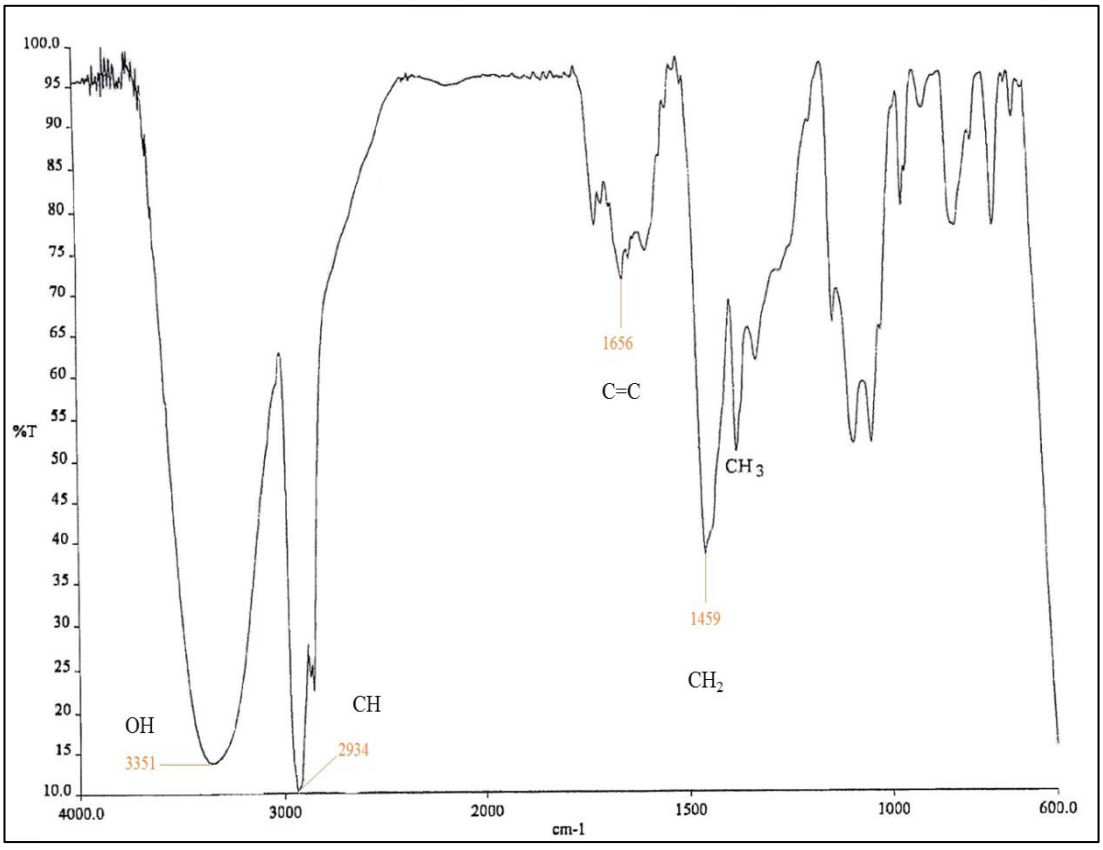


Figure 4.49: IR spectrum of stigmasterol (**60**)

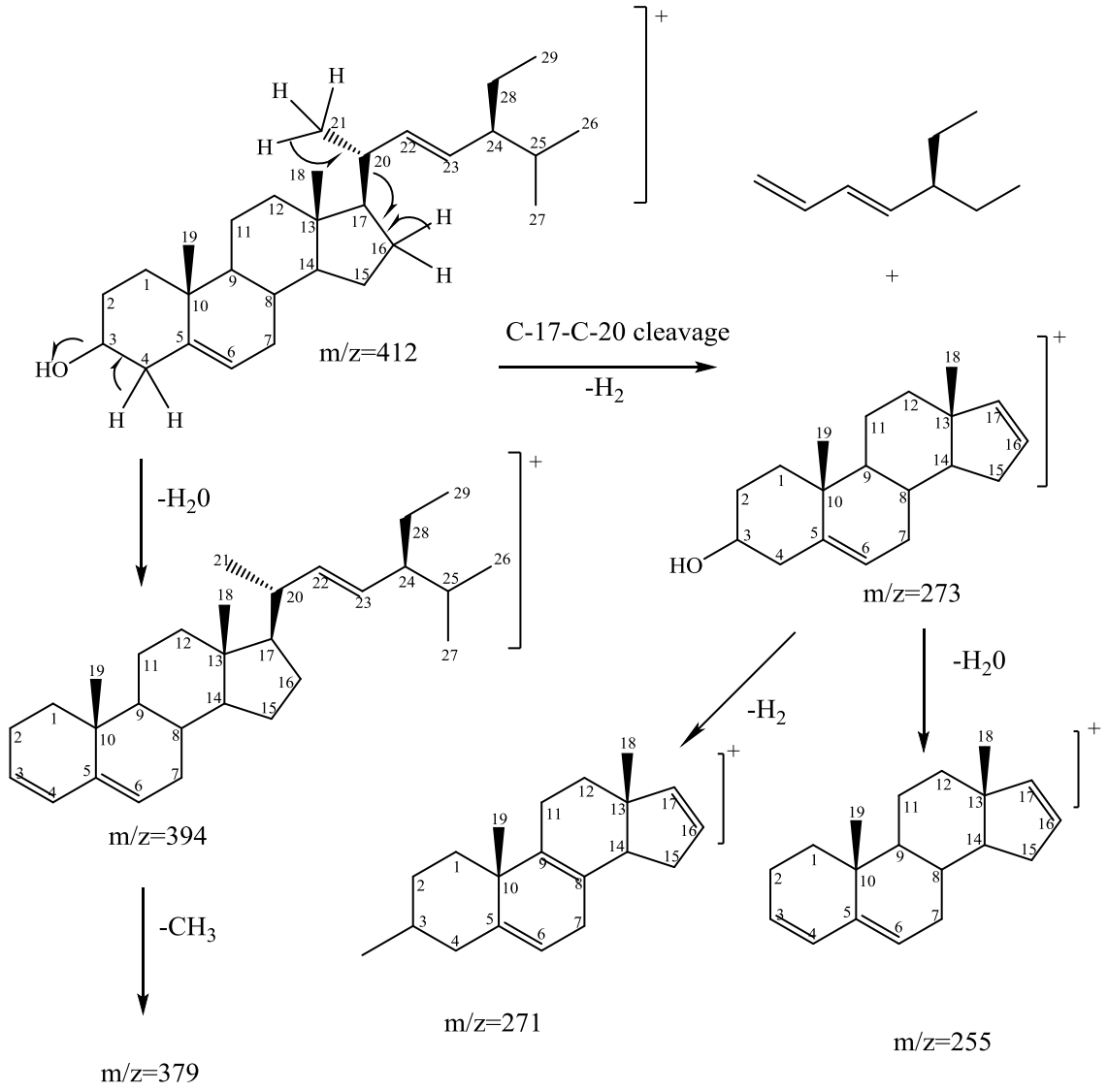


Figure 4.50: Mass fragmentation pattern of stigmasterol (**60**)

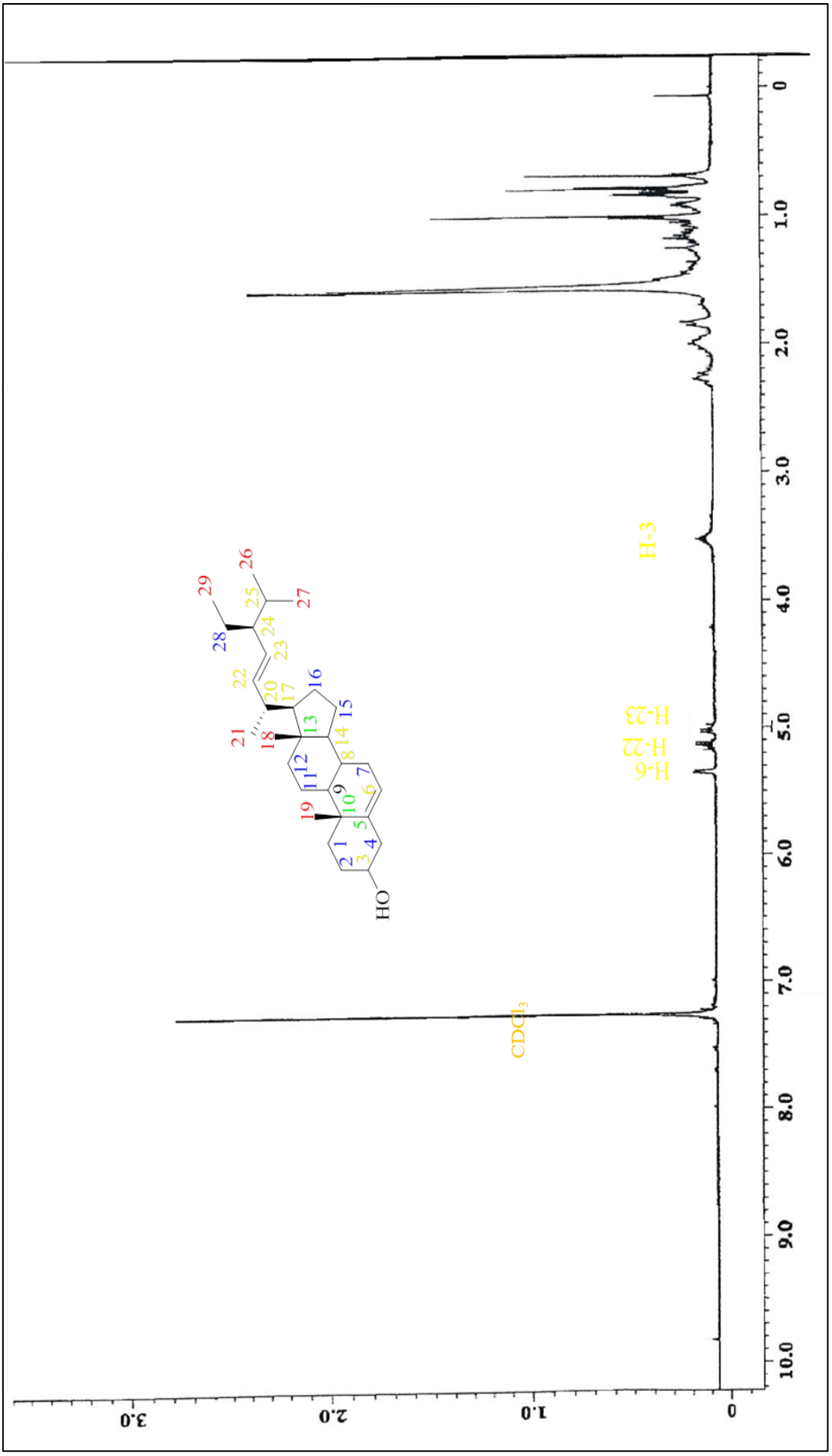


Figure 4.51: ^{1}H- NMR spectrum of stigmasterol (**60**)

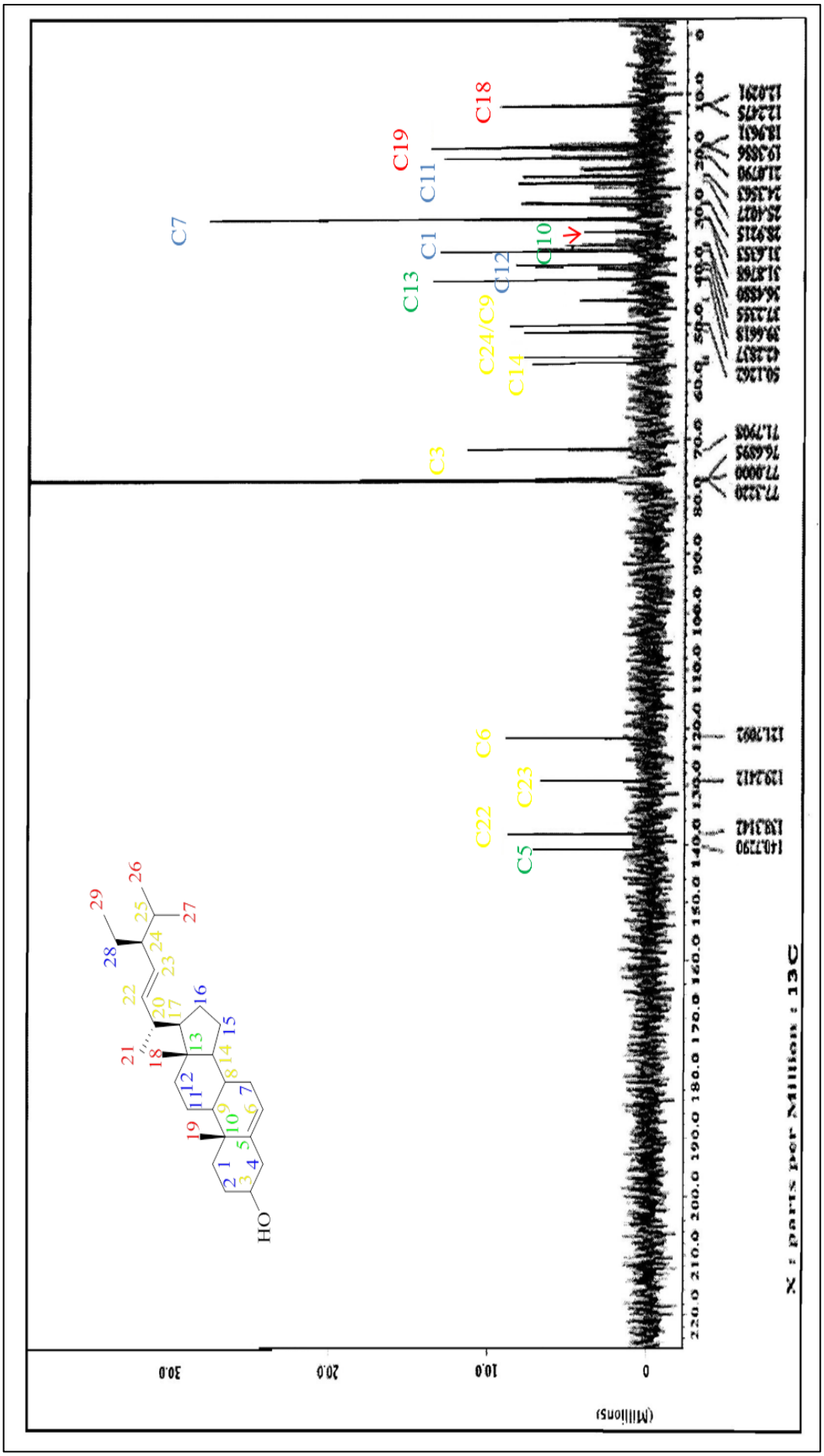
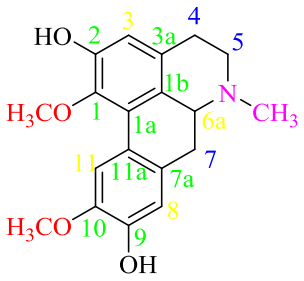


Figure 4.52: ^{13}C NMR spectrum of stigmasterol (**60**).

4.3.3 Boldine (5)



5

Alkaloid **5** was isolated from the dichloromethane extract and following acid-base extraction of the barks of *Phoebe grandis*. This alkaloid was obtained as brownish amorphous solid with $[\alpha]_D^{13}$ = +111° (c =0.5, C_2H_5OH) and the m.p 159-161°C [161°C reported from (Guinaudeau *et al*., 1975)]. By using high-resolution electrospray ionisation ESI^+ mass spectrometry, the spectrum (Figure 4.53) of alkaloid **5** gave a molecular ion peak at *m/z* 328.00 (100 %) $[M+1]^+$, suggesting a molecular formula of $C_{19}H_{21}NO_4$. The other significant peak was observed at *m/z* 265.08 (90 %).

The UV max value of alkaloid **5** showed absorption typical of aporphine at 220, 282 and 303 nm, respectively. These absorption peaks were due to the degree of resonance in the biphenyl system and indicated that these were aporphine substituted at positions 1,2,9 and 10 (Shamma & Slusarchyk, 1964; Sangster & Stuart, 1965; Guinaudeau *et al*., 1988). In addition, the IR spectrum (Figure 4.54) showed a broad band of hydroxyl stretching absorption at 3345 cm^{-1}. Absorption peaks at 2920 and 2845 cm^{-1} showed the presence of CH aromatic.

Furthermore, ^{1}H-NMR spectrum **(**Figure 4.55**)** showed the existence of the methoxyl groups by revealing two singlet peaks at δ 3.90 and 3.59 attached at position C-1 and C-10, respectively. The methoxyl protons at C-1 was observed rather shielded compared to the protons at C-10 since the protons of the methoxyl were force to place themselves

on top of ring D where the electron density was high. The N-methyl group resonated as a singlet at δ 2.54 and three aromatic singlets resonated at δ 6.64, 6.83 and 7.87 for H-3, H-8 and H-11, respectively. H-11 was found more deshielded due to the anisotropic effect caused by the ring A. The aliphatic protons of H-4, H-5 and H-7 appeared as multiplets at the region of δ 2.85-3.10. The position of C-10 methoxyl group was also proven by NOE difference experiment (Figure 4.56), which shows that irradiation of H-11 enhanced the methoxyl singlet at δ 3.59 and 3.90, thus confirming the position of OMe at C-1 and C-10.

The ^{13}C NMR spectrum **(**Figure 4.57**)** showed the presence of 19 carbon signals; three methylenes, four methines, nine quaternaries and three methyls. The chemical shift of three methylene carbons occured between δ 29.0 and 53.5. The four oxygenated aromatic quaternary carbons signals were observed at δ 142.2, δ 148.1, δ 145.2 and δ 145.7 indicated the presence of hydroxyl at C-2 and C-9, and methoxyl group at C-1 and C-10, respectively. One sp^3 C-6a appeared at δ 62.7. Moreover, the C-3 of alkaloid **5** resonated at δ 113.4 due to the fact that C-3 is *ortho* to a hydroxyl group. The hydroxyl group exhibited a lesser *ortho* shielding effect with respect to the methoxyl group. The two methoxyl carbons resonated at δ 56.3 (10-OCH_3) and δ 60.4 (1-OCH_3). On the basis of these spectral evidence and comparison (Table 4.9) with literature values (Guinaudeau *et al.,* 1975; Guinaudeau, *et. al.*, 1979; Lee & Yang, 1992; Ropi, *et. al.*, 2000), alkaloid **5** was characterised as boldine **(5)** or 1, 10-dimethoxyaporphine-2,9-diol or (4H-dibenzoquinoline-2,9-diol, 5, 6, 6a, 7-tetrahydro-1, 10-dimethoxy-6-methyl-) or 2,9-dihyroxy-1, 10-dimethoxy-aporphine.

Table 4.9: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of boldine (**5**) and the literature data.

	5 in $CDCl_3$		* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	142.2	-	142.0
1a	-	126.8	-	126.0
1b	-	126.0	-	125.0
2	-	148.1		148.0
3	6.64 (*s*, 1H)	113.4	6.63 (*s*, 1H)	113.3
3a		130.1	-	130.0
4	2.85-3.10 (*m*, 2H)	29.0	2.58-3.20 (*m*, 2H)	28.9
5	2.85-3.10 (*m*, 2H)	53.5	2.58-3.20 (*m*, 2H)	53.4
6a	2.85-3.10 (*m*, 1H)	62.7	2.58-3.20 (*m*, 1H)	62.6
7	2.85-3.10 (*m*, 2H)	34.3	2.58-3.20 (*m*, 2H)	34.2
7a	-	130.4	-	130.2
8	6.83 (*s*, 1H)	114.3	6.83 (*s*, 1H)	114.2
9	-	145.2	-	145.1
10	-	145.7	-	145.0
11	7.87 (*s*, 1H)	110.3	7.89 (*s*, 1H)	110.0
11a	-	123.8	-	123.0
1-OCH_3	3.59 (*s*, 3H)	60.4	3.60 (*s*, 3H)	60.2
10-OCH_3	3.90 (*s*, 3H)	56.3	3.91 (*s*, 3H)	56.1
NCH_3	2.54 (*s*, 3H)	44.1	2.52 (*s*, 3H)	44.0

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Ropi *et al*., 2000).

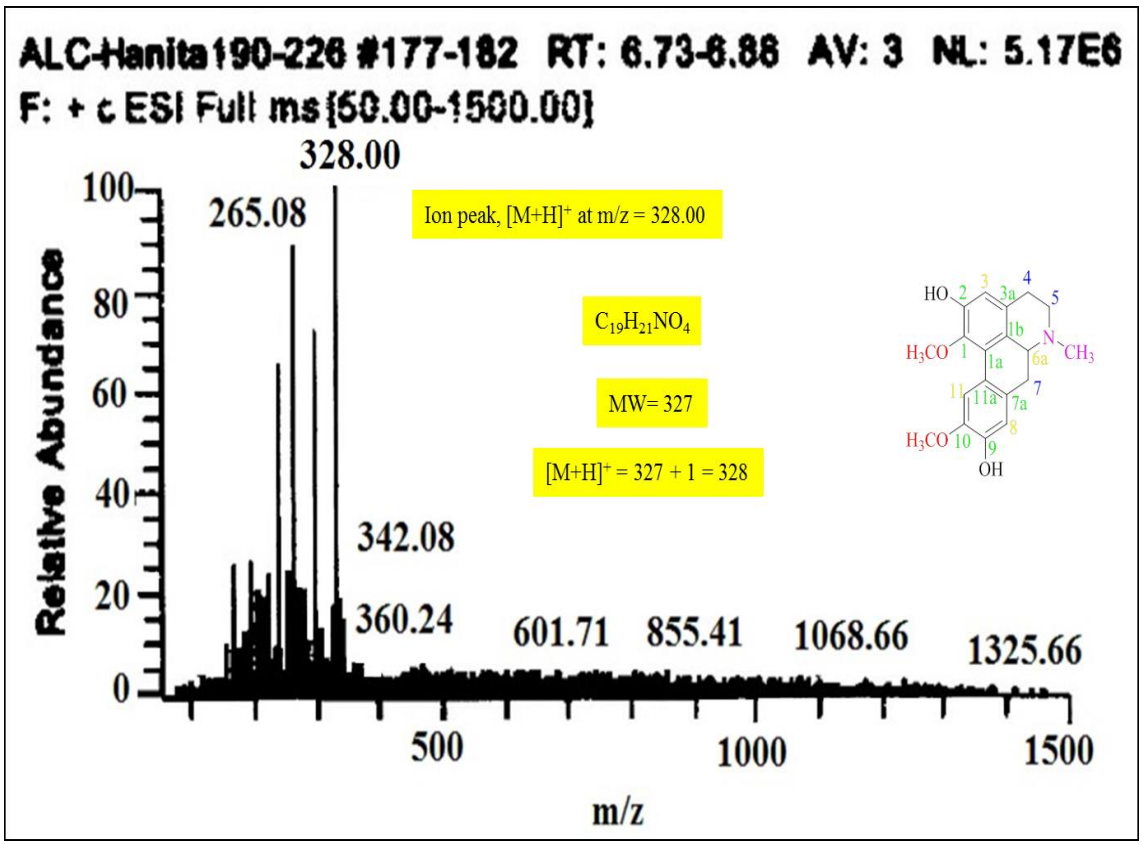


Figure 4.53: Electrospray Ionisation (ESI) Mass Spectrometry of boldine (**5**).

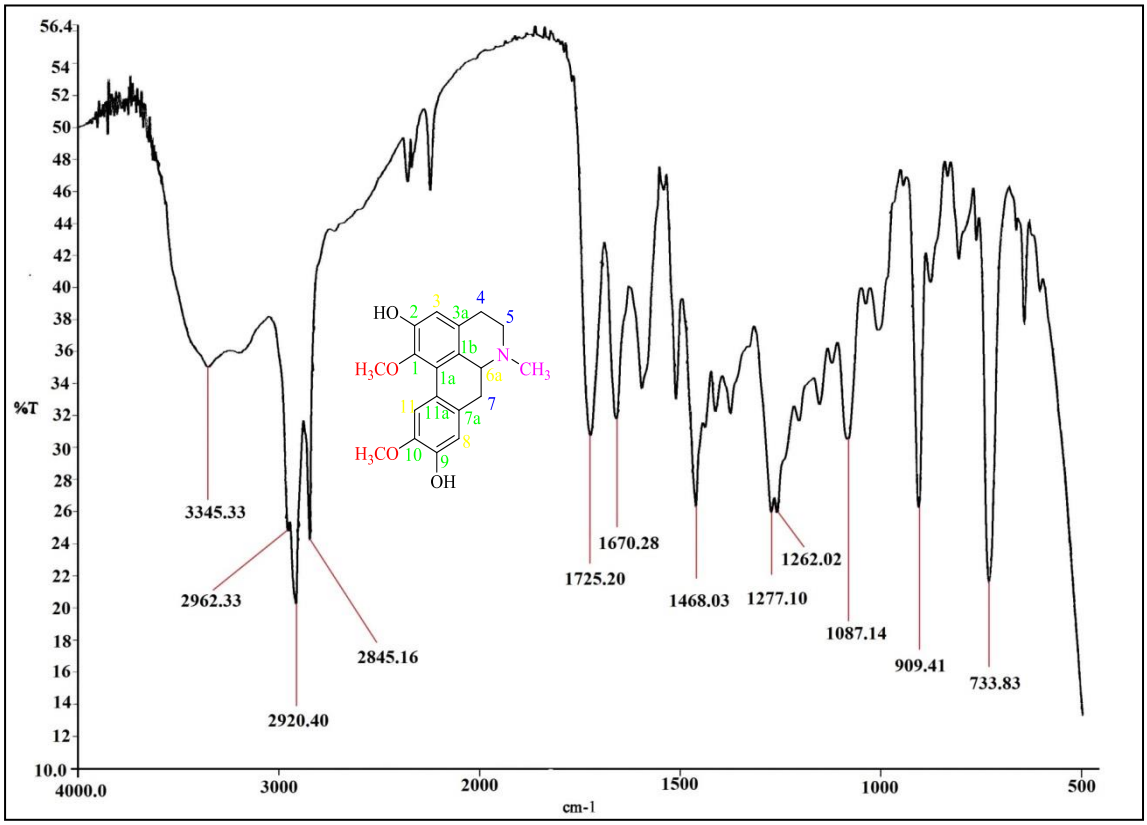


Figure 4.54: IR spectrum of boldine (**5**).

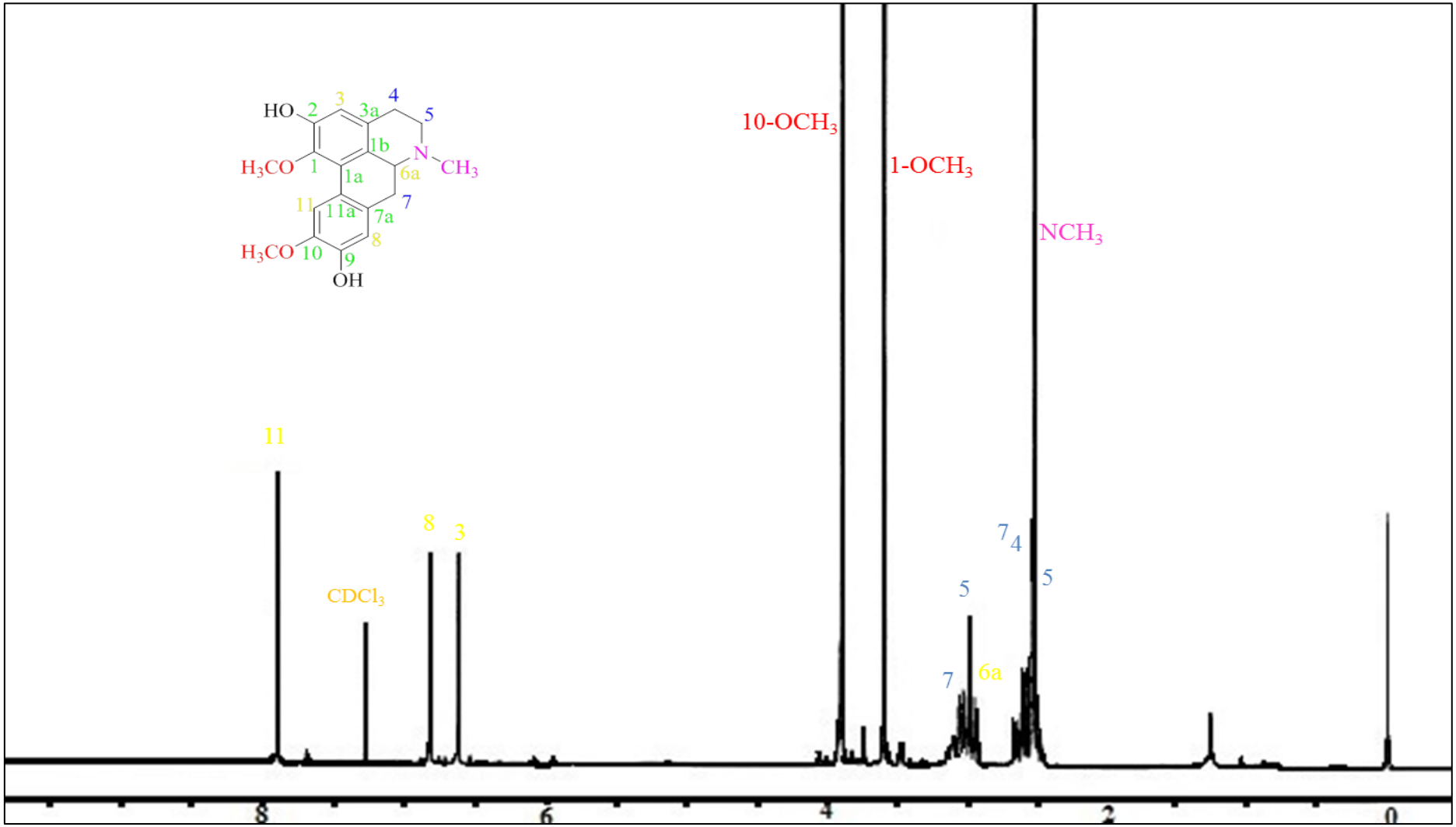


Figure 4.55: ^{1}H- NMR spectrum of boldine (**5**)

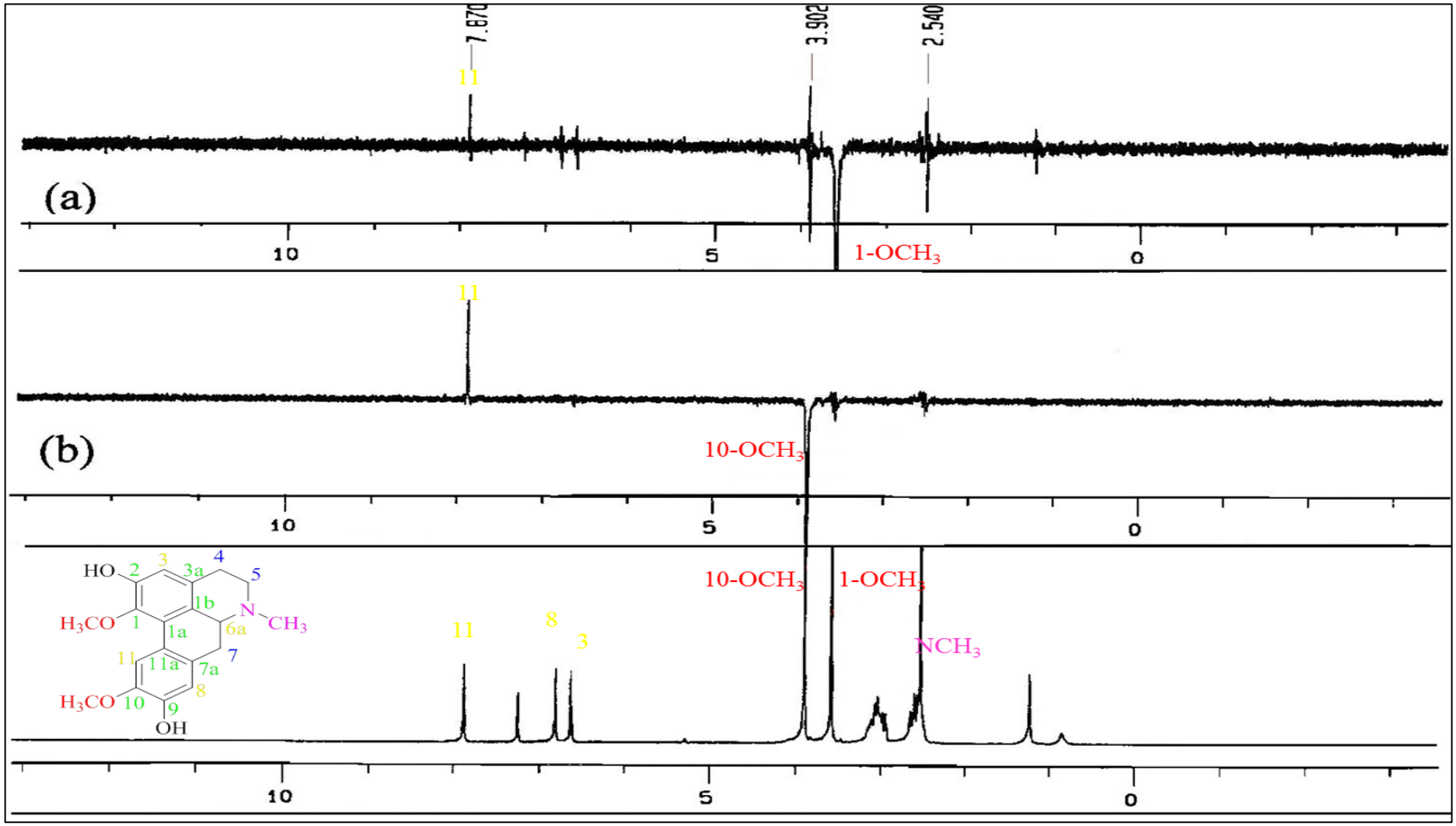


Figure 4.56: ^{1}H-NOE differential - NMR spectrum of boldine (5)

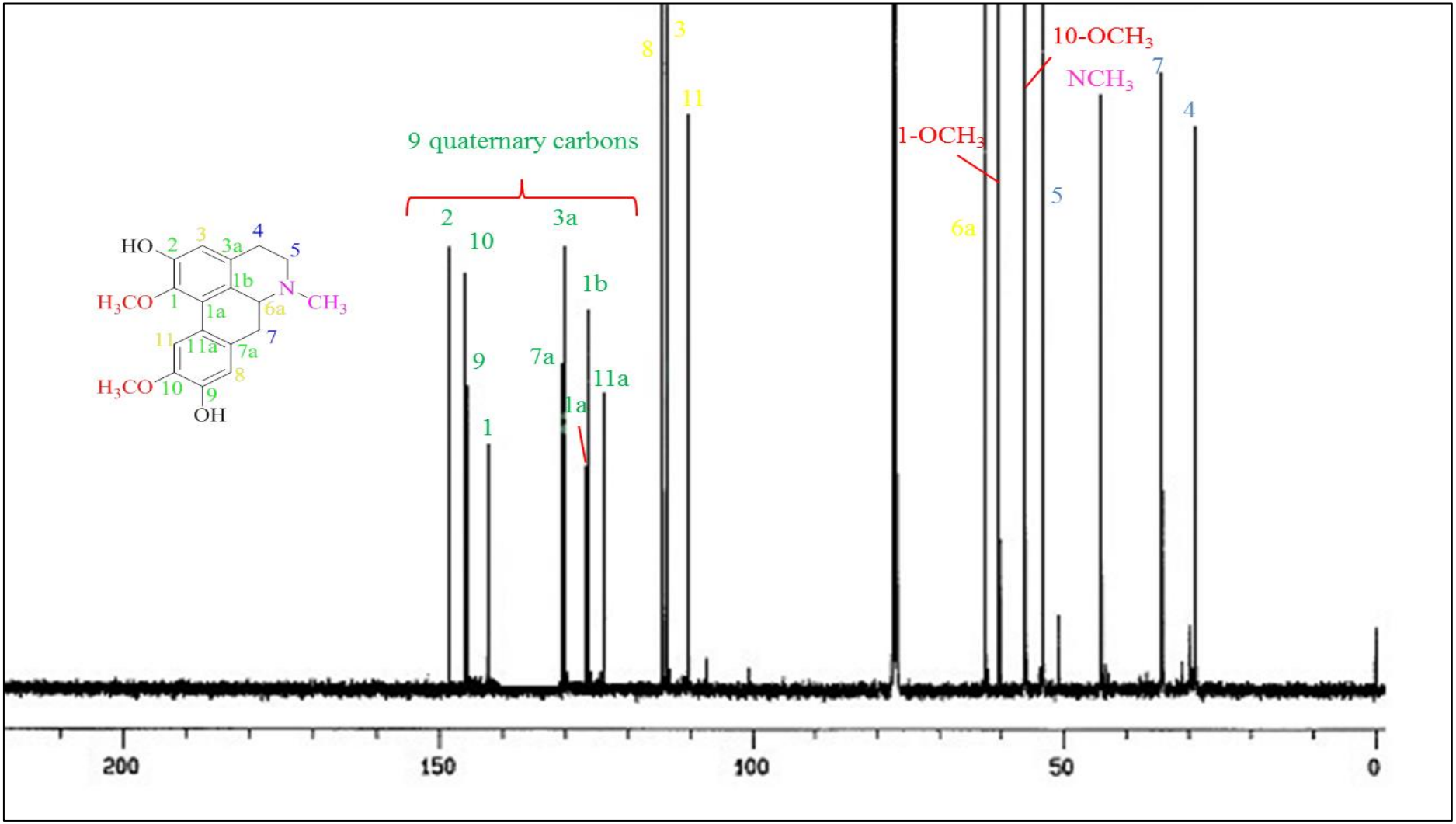
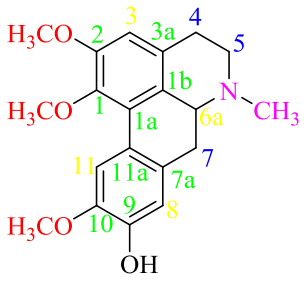


Figure 4.57: ^{13}C NMR spectrum of boldine (**5**)

4.3.4 N-methyllaurotetanine (51)



51

Alkaloid **51** was obtained as a yellow amorphous solid with $[\alpha]_D^{25}$ = +111° (c=1.0, MeOH). The HREIMS (Figure 4.58) exhibited an $[M+H]^+$ at m/z 342.1716 suggesting a molecular formula of $C_{20}H_{23}NO_4$ (calcd. for $C_{20}H_{24}NO_4$, 342.1705). The UV spectrum exhibit maxima at 215, 283 and 305 nm eliminated the possibility of a 9, 10-disubstitution. The IR spectrum (Figure 4.59) showed absorption peak at 3390 and 2935 cm^{-1} indicating a broad band of hydroxyl and the presence of CH aromatic, respectively.

The ^1H NMR spectrum (Figure 4.60) of **51** gave evidence for the presence of seven singlet signals belong to three aromatic protons, three methoxyl and a methyl group attached to the nitrogen atom. Three singlets at δ 6.58, 6.85 and 8.04 were assigned to H-3, H-8 and H-11, respectively. These signals are typical of 1,2,9,10-tetrasubstituted aporphines (Shamma, 2012). Three methoxyl signals were present at δ 3.62, 3.86 and 3.89 which belongs to C-1, C-2 and C-10, respectively. The singlet at δ 3.62 is assigned to the methoxyl at C-1 since the protons were shielded by the anisotropic effect caused by the ring D. The downfield signal of H-11 at δ 8.04 suggested that C-1 was substituted by a methoxyl group. The N-methyl group appeared as singlet at δ 2.54. The spectrum also showed the resonances of doublet of doublets (*dd, J*=4.3, 15.5 Hz which attributable to H4α and H4β. The other aliphatic protons H5 also appeared as doublet of

doublets and the H7 appeared as doublet occurred in the range of δ 3.20-2.50. These ^{1}H NMR spectra data were also supported by the COSY spectrum (Figure 4.63)

The ^{13}C NMR spectra (Fig. 4.61) showed the presence of twenty carbon atoms including nine quaternary carbon signals which validated the molecular formula $C_{20}H_{23}NO_4$. The DEPT 135 experiment (Fig. 4.62) showed three methoxyl, one N-methyl, three methylenes and one methine. The chemical shift values of the ^{13}C NMR carbon signals are given in the Table 4.10.

The COSY spectrum (Figure 4.63) showed that H-4 was only correlated with H-5, while H -7 showed an interaction with H-6a. The above observations were reinforced by NOESY experiment (Figure 4.64) which showed correlations between H-6a/H7, H3/H4, H8/H7, H3/2-OMe, H11/1-OMe and H11/10-OMe.

The ^{1}H-^{13}C direct correlations were determined by using HSQC spectrum (Figure 4.65) and the results were supported by other data. The structure was finally confirmed by ^{1}H-^{13}C long range correlations in the HMBC spectrum (Figure 4.66). The assignment of 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR has been summarised in Table 4.10 since 2D NMR data were not reported in the previous literature.

Based on the above analysis data, the structure of alkaloid **51** was deduced as N-methyllaurotetanine (**51**) by comparison of its spectral data with literature values (Wu *et al.*, 1980; Lee & Yang, 1992; Zhang *et al*., 2010). Interestingly, N-methyllaurotetanine (**51**) is reported here for the first time as naturally present in *Phoebe grandis*.

Table 4.10: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) data and 2D (HMBC and HSQC) NMR data of N-methyllaurotetanine (**51**) and the literature data.

	51 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^{2}J,^{3}J)	HSQC (^{1}J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	144.2	-	-	-	144.9
1a	-	127.1	-	-	-	126.6
1b	-	127.2	-	-	-	126.9
2	-	152.0	-	-	-	151.9
3	6.58 (*s*, 1H)	110.3	C_{1b}, C_4, C_1, C_2	H_3	6.58 (*s*, 1H)	110.0
3a	-	129.0	-	-	-	128.6
4	β 3.15 (*dd*, *J*= 4.3, 15.5) α 2.66 (*dd*, *J*= 4.3, 15.5)	29.3	C_5, C_{3a}	$2H_4$		28.8
5	β 3.06 (*dd*, *J*= 6.4, 12.4) α 2.48 (*dd*, *J*= 6.4, 12.4)	53.3	C_4, C_{6a}, C_{3a}	$2H_5$		53.1
6a	2.99 *m*	62.6	N-Me, C_8	H6a		62.4
7	β 2.95 (*d*, *J*=4.1) α 2.51 (*d*, *J*=4.1)	34.3	C_8, C_{11a}, C_{1b}, C_{7a}	$2H_7$		33.9
7a	-	130.2		-	-	128.7
8	6.85 (*s*, 1H)	114.0	C_7, C_{11a}, C_{10}	H8	6.81 (*s*, 1H)	114.1
9	-	144.9	-	-	-	145.4
10	-	145.3	-	-	-	144.0
11	8.04 (*s*, 1H)	111.2	C_{3a}, C_9	H11	8.06 (*s*, 1H)	111.1
11a	-	124.0	-	-	-	123.6
1-OMe	3.62 (*s*, 3H)	60.2	C_1	$3H_{1\text{-OMe}}$	3.65 (*s*, 3H)	60.0
2-OMe	3.86 (*s*, 3H)	55.9	C_2	$3H_{2\text{-OMe}}$	3.88 (*s*, 3H)	55.7
10-OMe	3.89 (*s*, 3H)	56.1	C_9	$3H_{10\text{-OMe}}$	3.89 (*s*, 3H)	55.9
N-Me	2.54 (*s*, 3H)	44.0	C_5, C_{6a}	$3H_{N\text{-Me}}$	2.55 (*s*, 3H)	43.7

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.
* (Zhang *et al*., 2010).

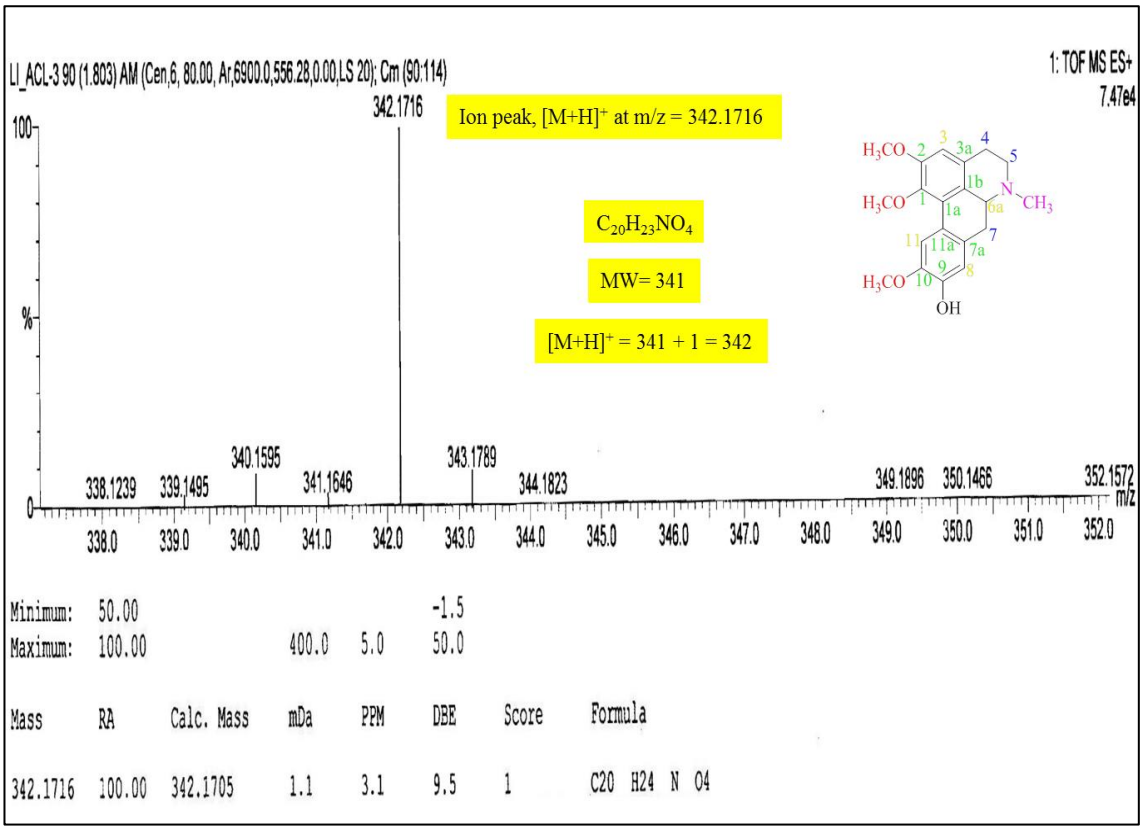


Figure 4.58: HREIMS spectrum of N-methyllaurotetanine (**51**).

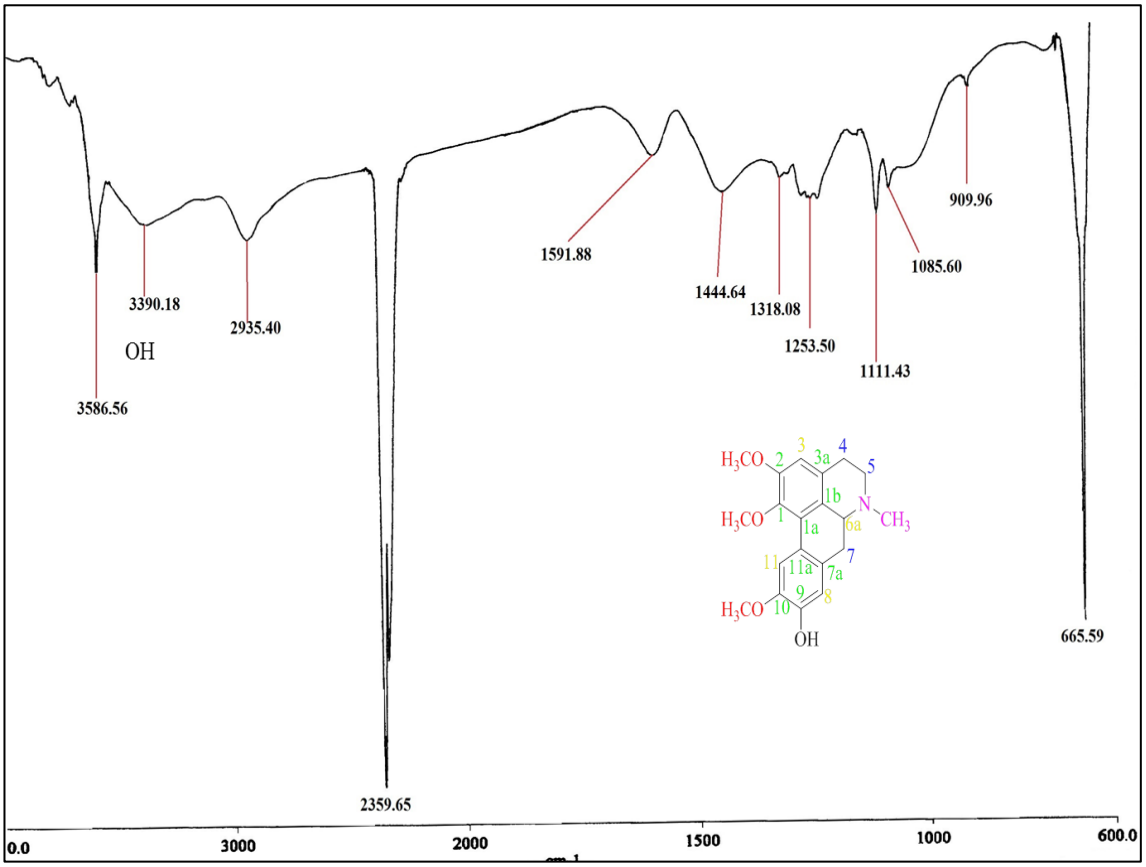


Figure 4.59: IR spectrum of N-methyllaurotetanine (**51)**

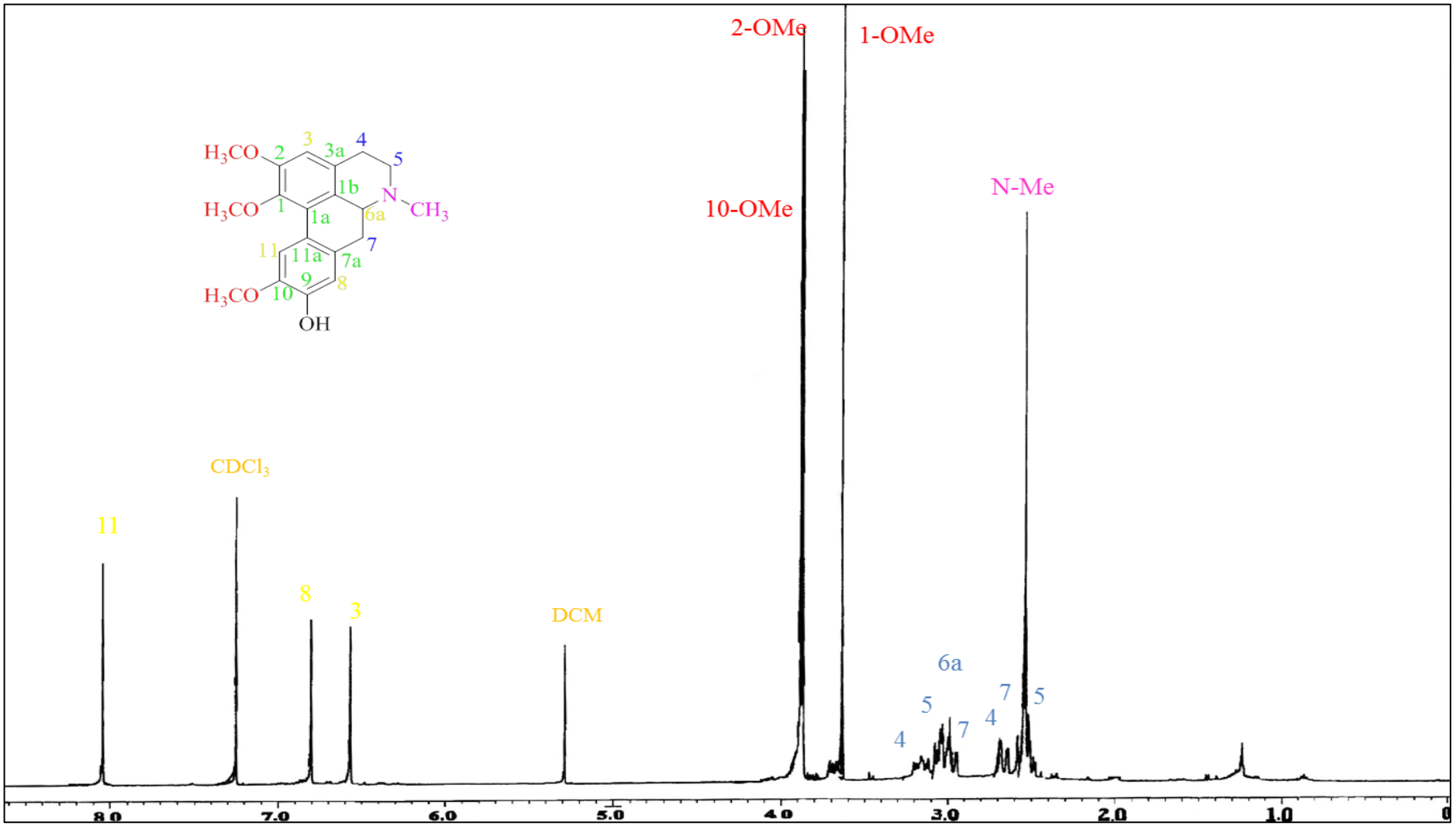


Figure 4.60: ^{1}H- NMR spectrum of N-methyllaurotetanine (**51**)

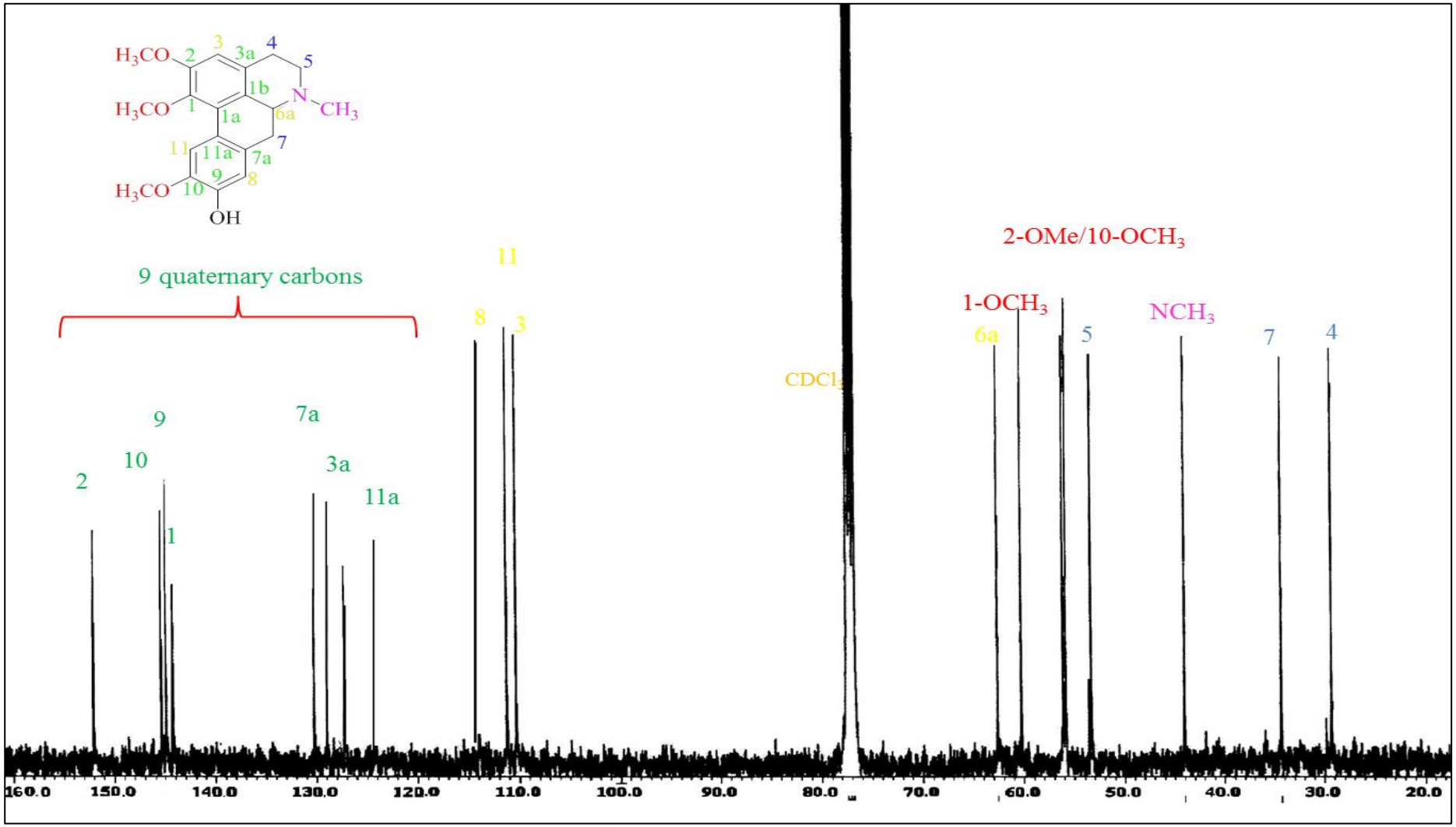


Figure 4.61: ^{13}C-NMR spectrum of N-methyllaurotetanine (**51**).

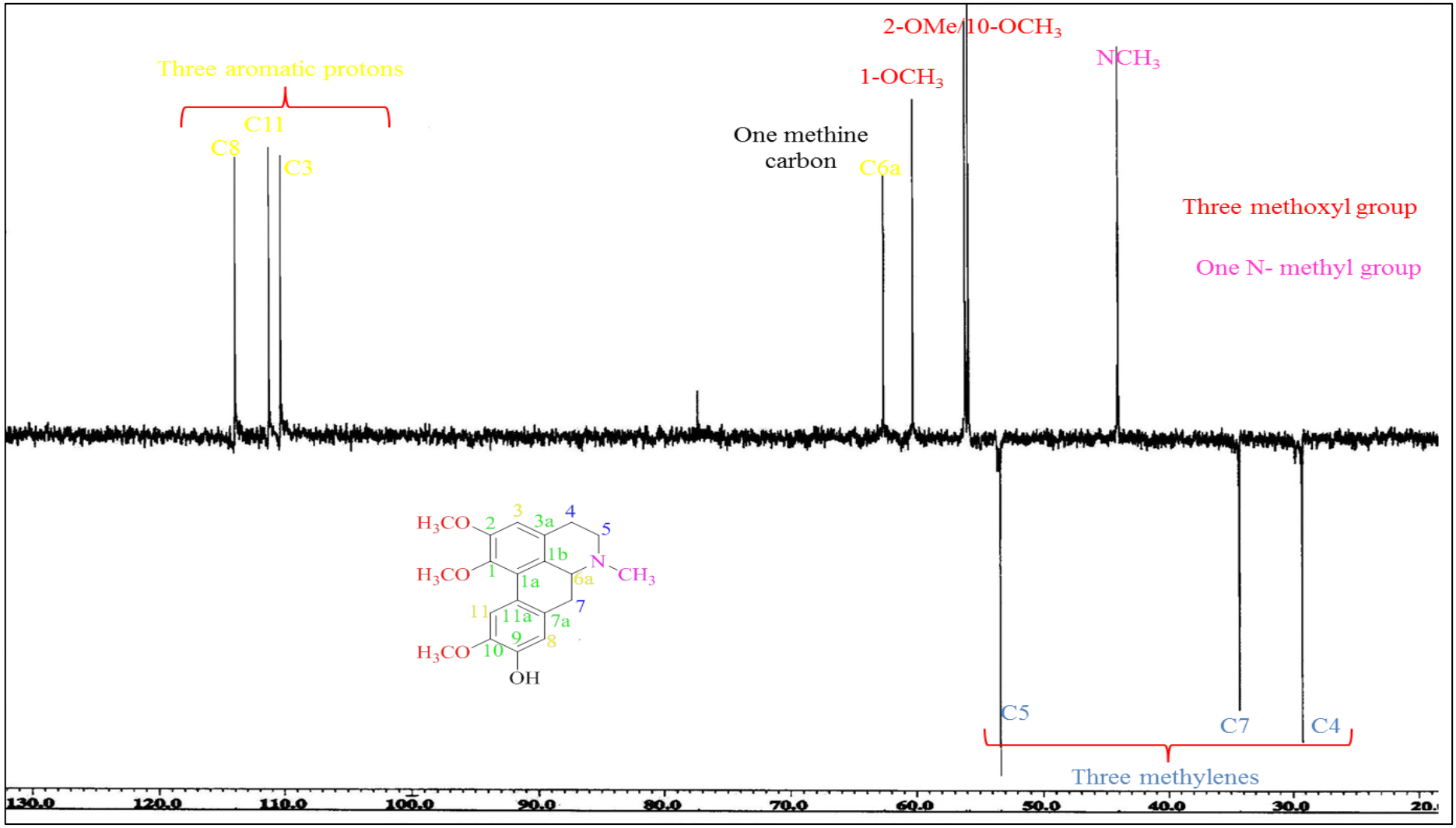


Figure 4.62: DEPT spectrum of N-methyllaurotetanine (**51**).

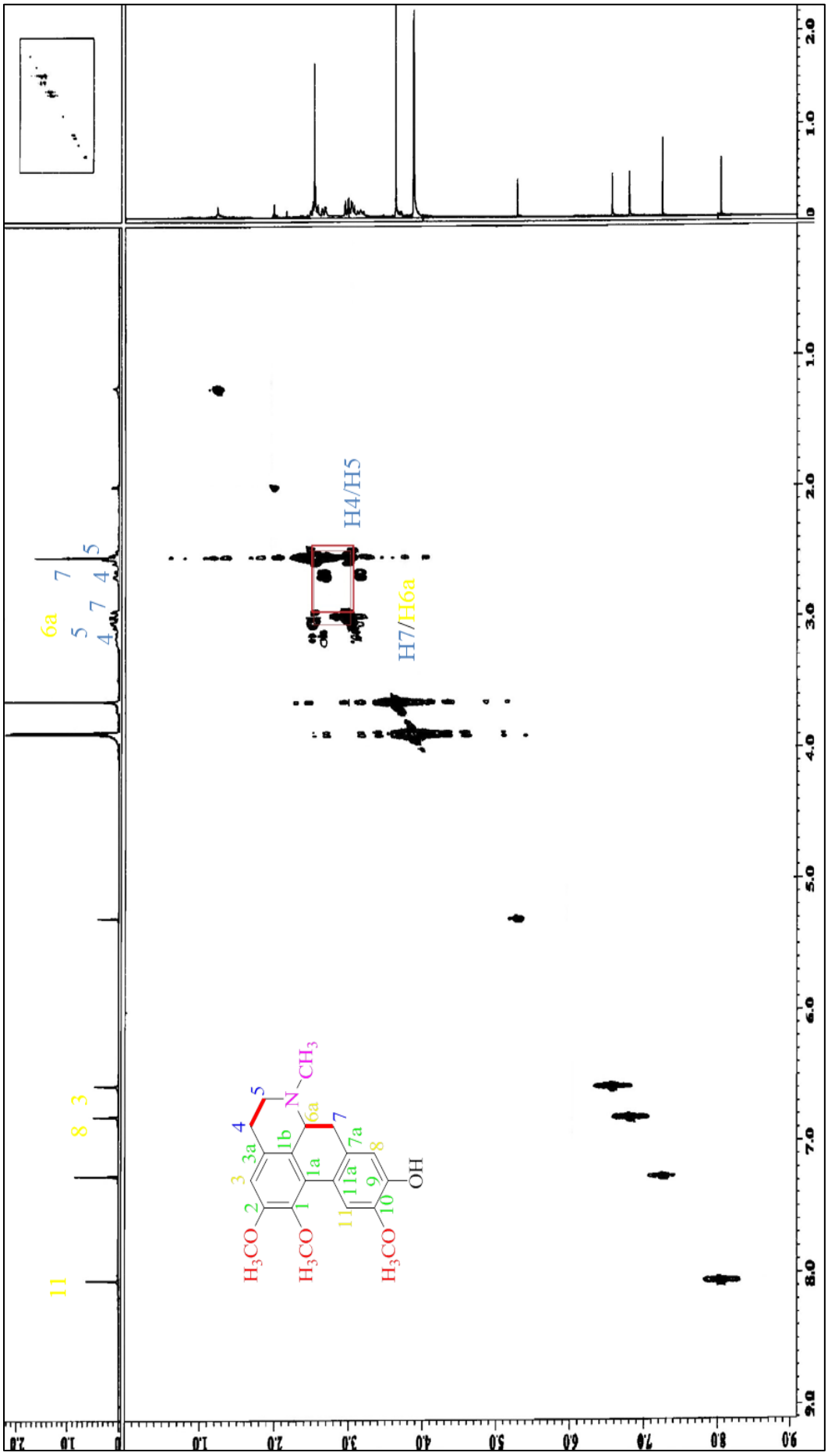


Figure 4.63: COSY spectrum of N-methyllaurotetanine (**51**).

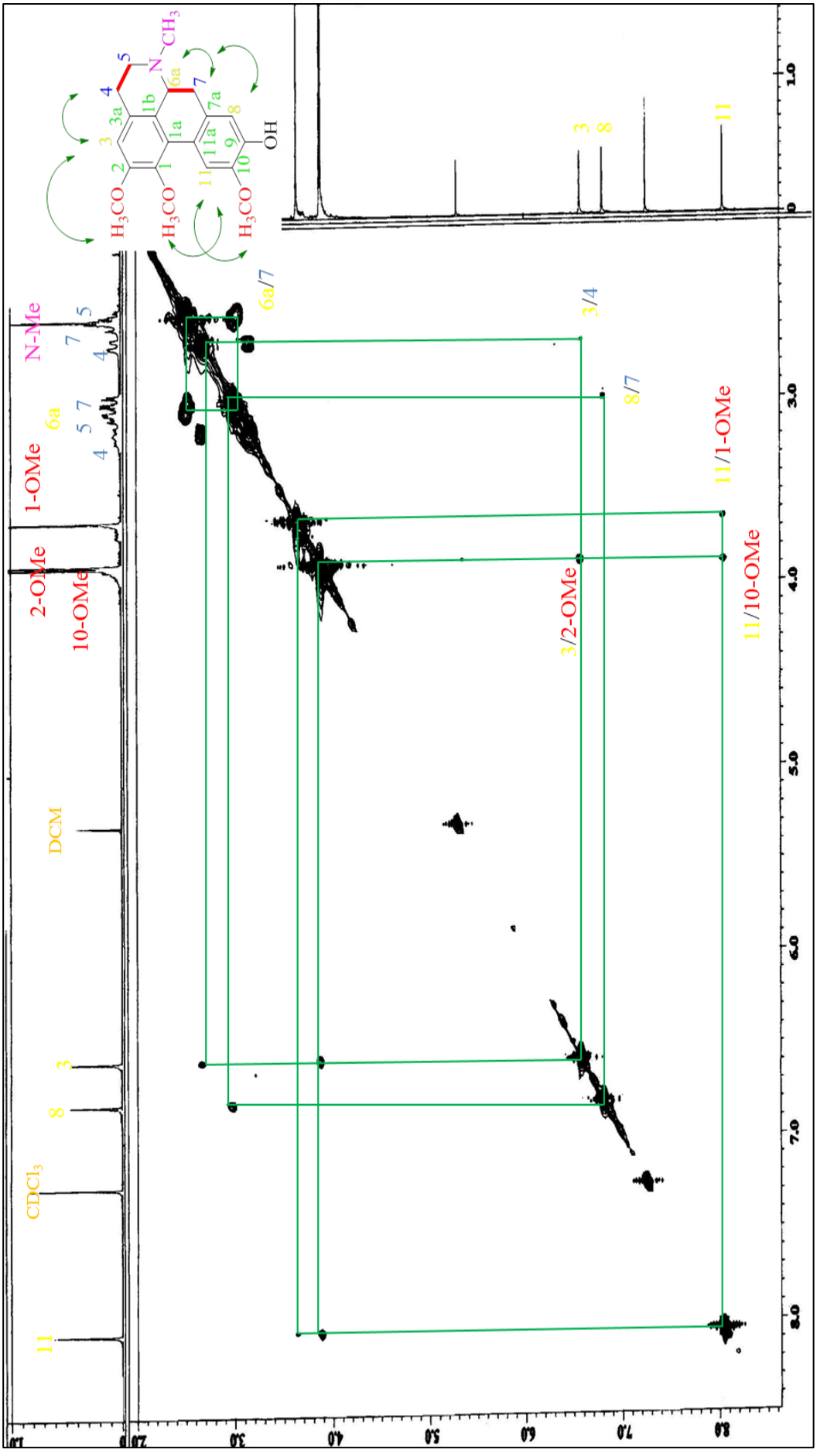


Figure 4.64: NOESY spectrum of N-methyllaurotetanine (**51**).

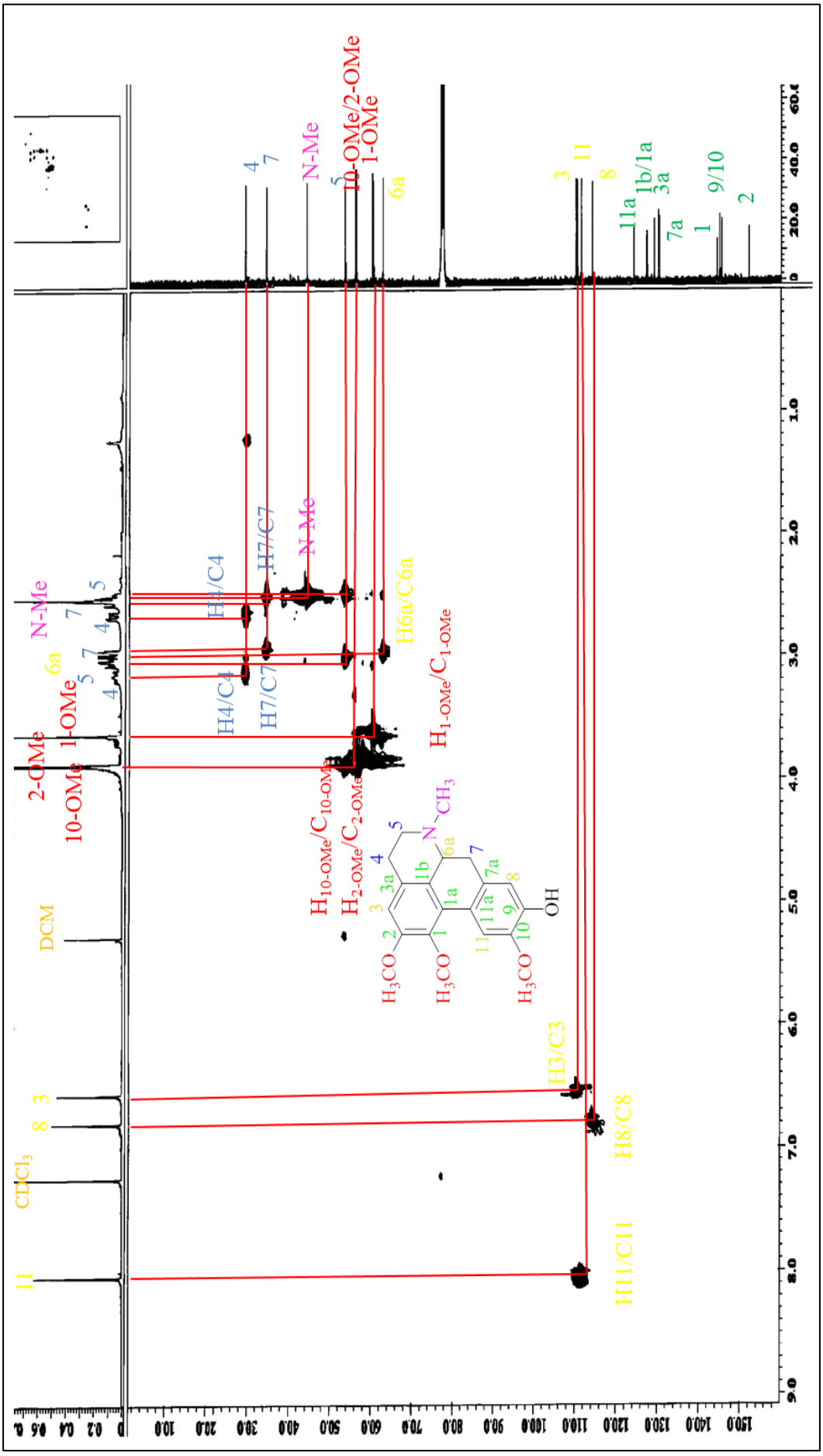


Figure 4.65: HSQC spectrum of N-methyllaurotetanine (**51**).

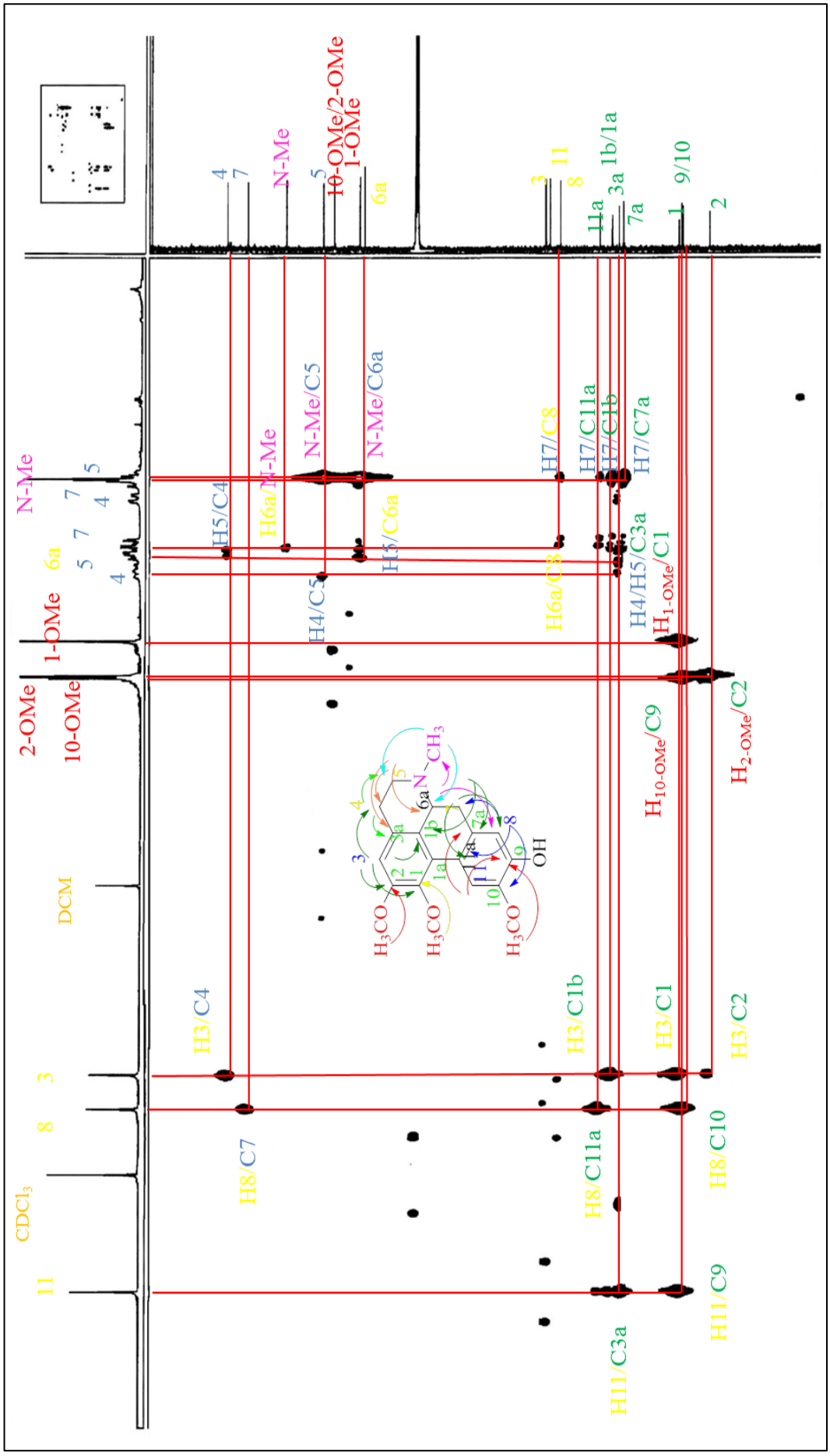
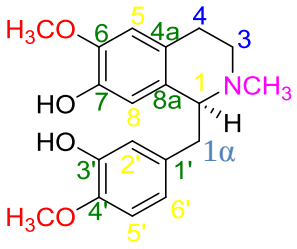


Figure 4.66: HMBC spectrum of N-methyllaurotetanine (**51**).

4.3.5 Reticuline (61)



61

Alkaloid **61**, $[\alpha]_D^{25}$ = + 0.003° (c=0.5, MeOH), was obtained as brownish amorphous solid. The LCMS -Q TOF spectrum (Figure 4.67) showed the presence of a molecular ion peak at m/z 330.1695 $[M+H]^+$, thus suggesting a molecular formula of $C_{19}H_{23}NO_4$ (calcd for $C_{19}H_{24}NO_4$, 330.1661). The UV spectrum showed absorption band at 235 and 283 nm which were a characteristic of a benzylisoquinoline alkaloid. Meanwhile, the IR spectrum showed absorption at 3392 cm^{-1} and 2918 cm^{-1} due to the stretching of OH and CH aromatic.

The ^1H NMR spectrum (Figure 4.68) exhibited five aromatic protons, two methoxyl groups and a methyl group attached to the nitrogen atom. Two singlets appeared at δ 6.30 and δ 6.50 were assigned to H8 and H5, respectively. The former was shifted to the upfield region due to the shielding effect by the ring C. The spectrum also showed the resonances of three protons in ring C; H2', H5' and H6'. Two of doublets at δ 6.77 (*d*, *J*=1.92 Hz, 1H) and δ 6.68 (*d*, *J*=8.04 Hz, 1H) were assigned to H2' and H5', respectively. Meanwhile, a doublet of doublets (*dd*, *J*=8.04, *J'*=1.9 Hz) attributed to H6' was observed at δ 6.51. Two methoxyl signals were present at δ 3.84 and 3.80 belong to 6-OCH_3 and 4'-OCH_3, respectively. One N-methyl proton resonated as a singlet at δ 2.44. The aliphatic protons appeared as multiplets at the region between δ 2.50 - δ 3.66 attributable to the aliphatic protons of H1α, H1, H3 and H4 which gave the total of seven protons.

The ^{13}C NMR spectrum (Figure 4.69) showed the presence of nineteen carbon atoms which validated the molecular formula of $C_{19}H_{23}NO_4$. The methoxyl carbon signals appeared at δ 55.82 and 55.91. While the DEPT spectrum (Figure 4.70) indicated the presence of three methylene carbons, one methyne, one methyl carbon attached to a nitrogen atom, five aromatic carbons, seven quaternary carbons and two methoxyl carbons in the molecule skeleton.

The COSY spectrum (Figure 4.71) showed that H5' was only correlated with H6', but in shielded area, H-3α correlated with H-4α, H-3β correlated with H-4β, H-4β correlated with H-3β and H-4α correlated with H-3α.

The complete assignment of carbon and hydrogen in the structure was further confirmed by HSQC (Figure 4.72) and HMBC (Figure 4.73) experiments and were tabulated in Table 4.11. Finally, unambiguous assignment of all proton and carbon signals using HMBC, HSQC and COSY and by comparison with literature data showed that the alkaloid **61** was deduced as reticuline (**61**), which was isolated as natural source for the first time to this species *Phoebe grandis*.

Table 4.11: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) data and 2D (HMBC and HSQC) NMR data of reticuline (**61**) and the literature data.

	61 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^{2}J,^{3}J)	HSQC (^{1}J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	3.66 (*t*, *J*=6.0)	64.4	N-CH_3, C3, C8, C4a, C1', C8a	H1	3.63-3.66 *m*	64.6
3	2.80-3.20 *m*	46.4	C4a	2H3	2.69-3.18 *m*	46.7
4	2.50-2.85 *m*	24.7	C3, C4a	2H4	2.53-2.82 *m*	24.9
4a	-	129.7	-	-	-	129.9
5	6.50 (*s*, 1H)	110.6	C4, C8a, C7	H5	6.52 (*s*, 1H)	110.5
6	-	145.3	-	-	-	145.1
7	-	143.5	-	-	-	143.4

8	6.30 (*s*, 1H)	114.0	C-1, C8a, C6	H8	6.38 (*s*, 1H)	113.7
8a	-	124.7	-	-	-	125.1
1α	2.75-3.05 *m*	40.8	C1, C2', C6', C1', C8a	2H1α	2.69-3.02 *m*	41.0
1'	-	132.8	-	-	-	133.0
2'	6.77 (*d*, J_m=1.9)	115.8	C1α, C6', C4'	H2'	6.75 (*d*, J_m=1.9)	115.6
3'	-	145.4	-	-	-	145.2
4'	-	145.5	-	-	-	145.3
5'	6.68 (*d*, *J*=8.0)	110.7	C1', C3'	H5'	6.71 (*d*, *J*=8.3)	110.6
6'	6.51 (*dd*, J_o=8.0, J_m=1.7)	121.0	C1α, C2', C4'	H6'	6.57 (*dd*, J_o=8.0, J_m=1.9)	121.0
6-OCH_3	3.84 (*s*, 3H)	55.8	C6	-	3.83 (*s*, 3H)	55.9
4'-OCH_3	3.80 (*s*, 3H)	55.9	C4'	-	3.83 (*s*, 3H)	55.9
N-Me	2.44 (*s*, 3H)	42.1	C1, C3	-	2.43 (*s*, 3H)	42.4

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.
* (Mollataghi, 2012).

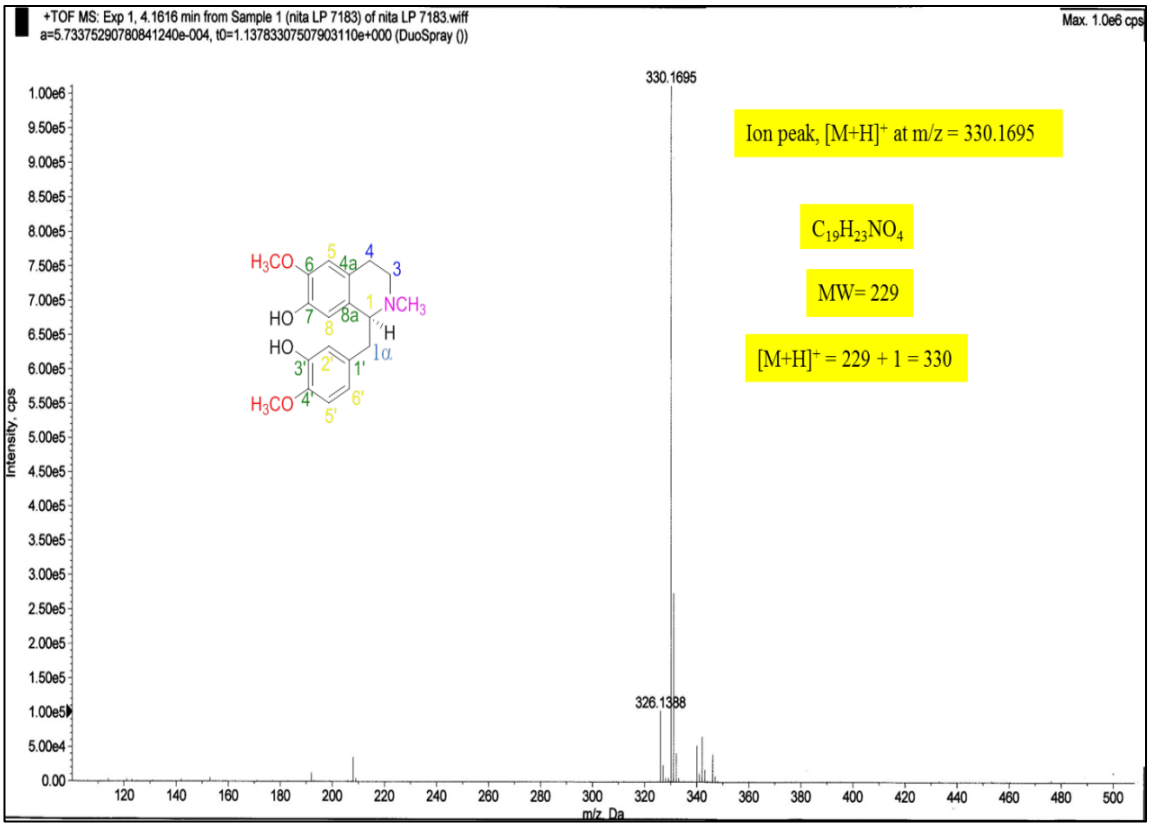


Figure 4.67: LCMS- Q TOF spectrum of reticuline (**61**)

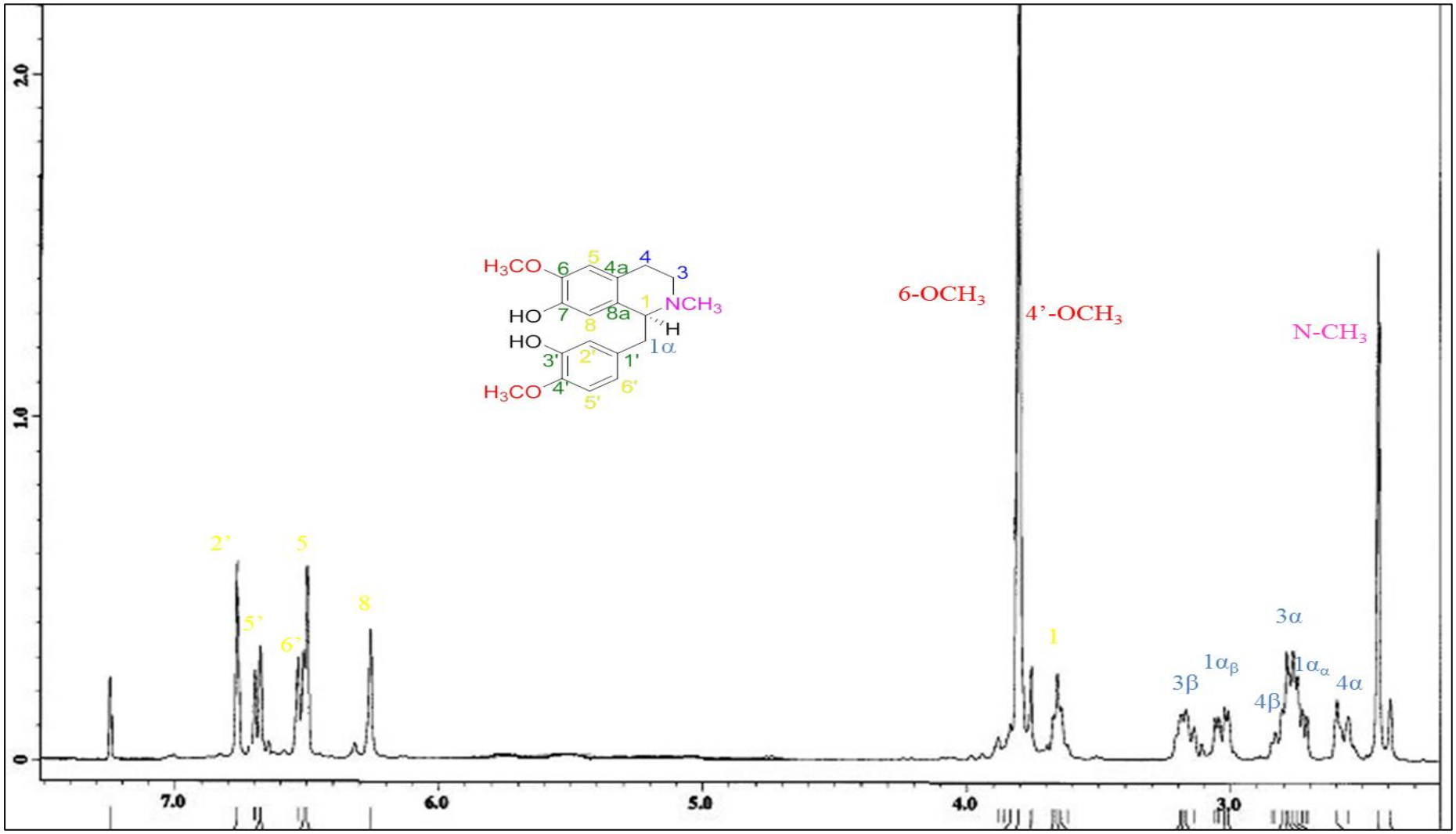


Figure 4.68: ^{1}H- NMR spectrum of reticuline (**61**)

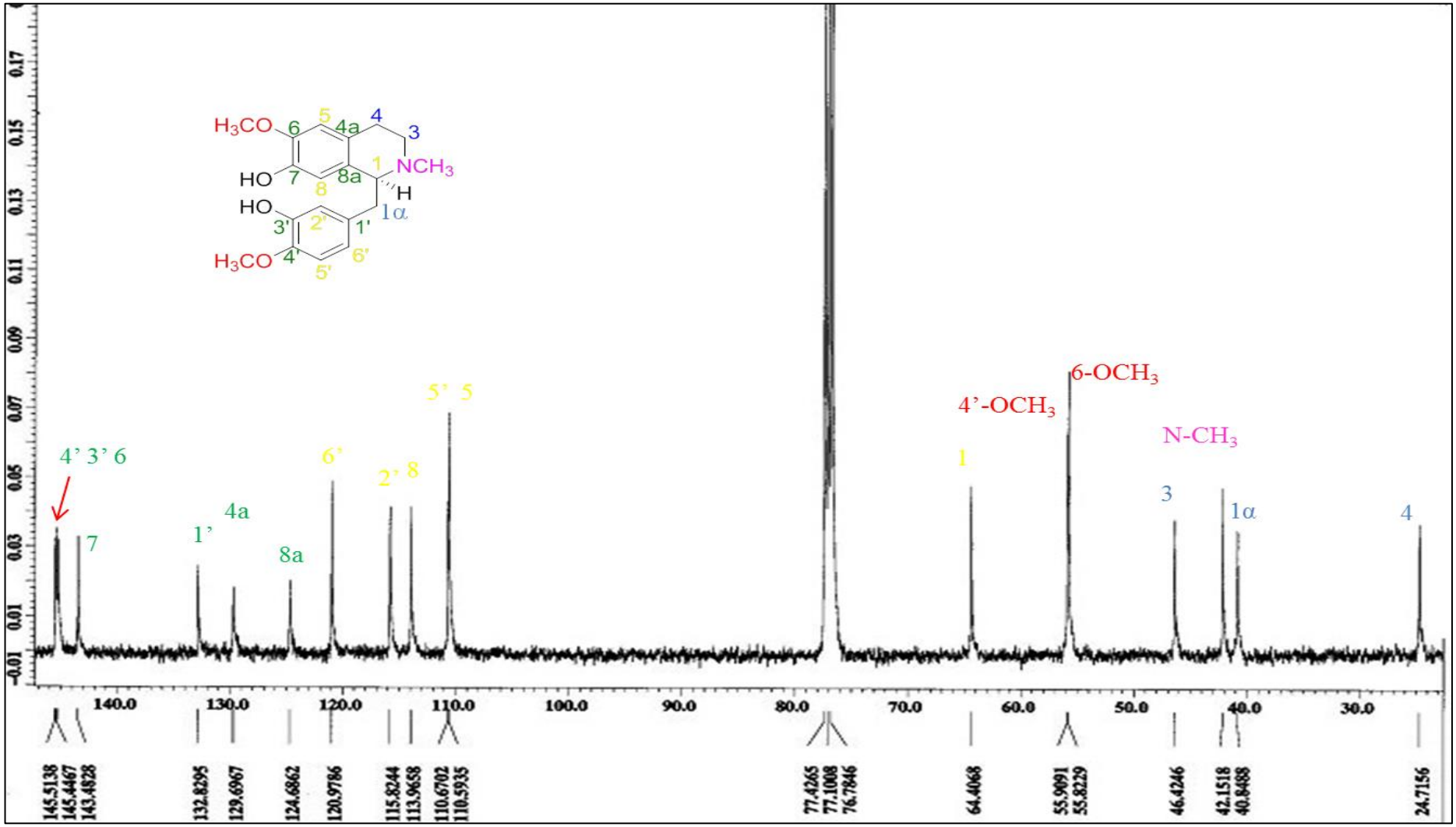


Figure 4.69: ^{13}C- NMR spectrum of reticuline (**61**)

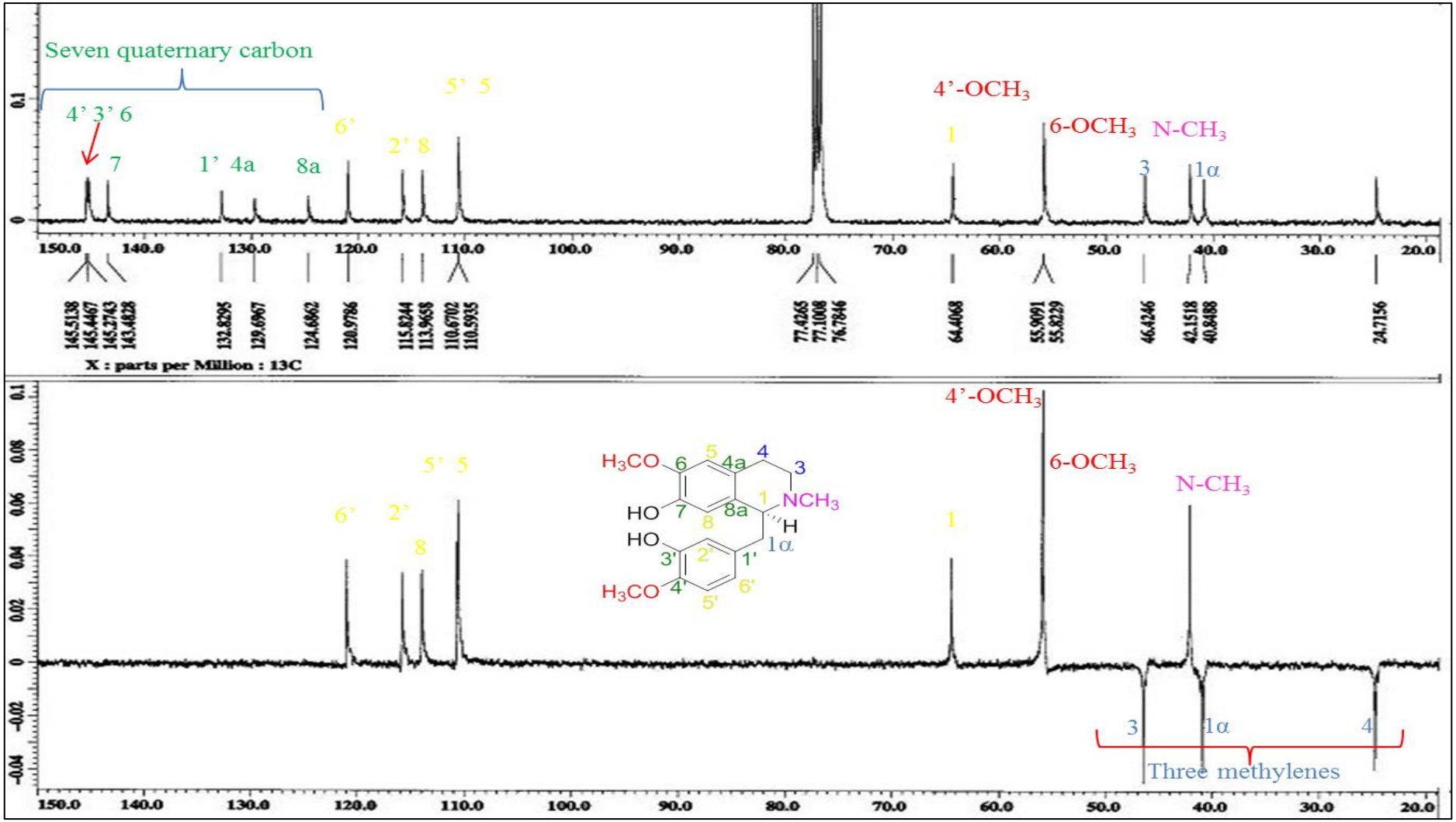


Figure 4.70: DEPT spectrum of reticuline (**61**)

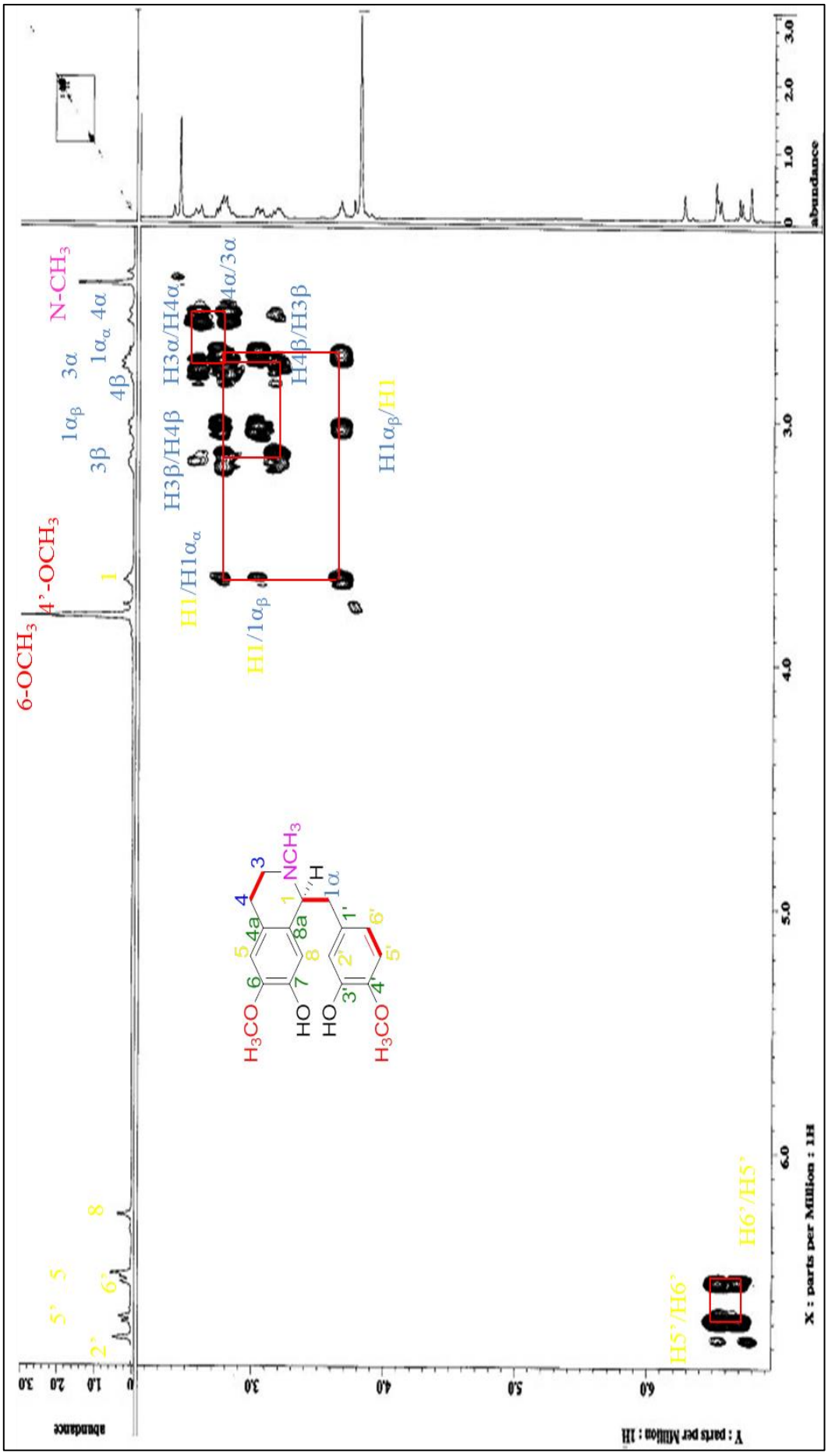


Figure 4.71: COSY spectrum of reticuline (**61**)

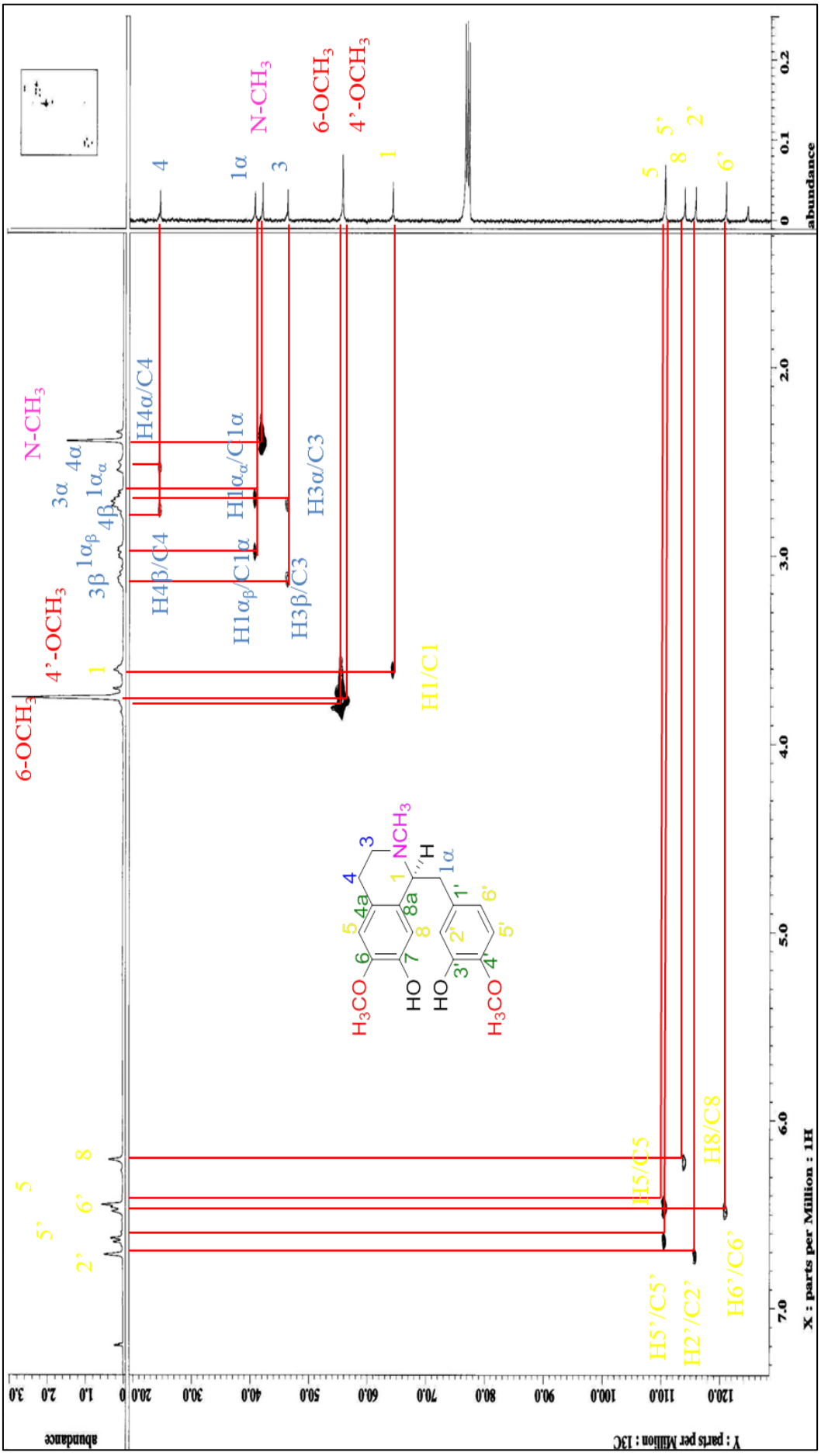


Figure 4.72: HSQC spectrum of reticuline (**61**)

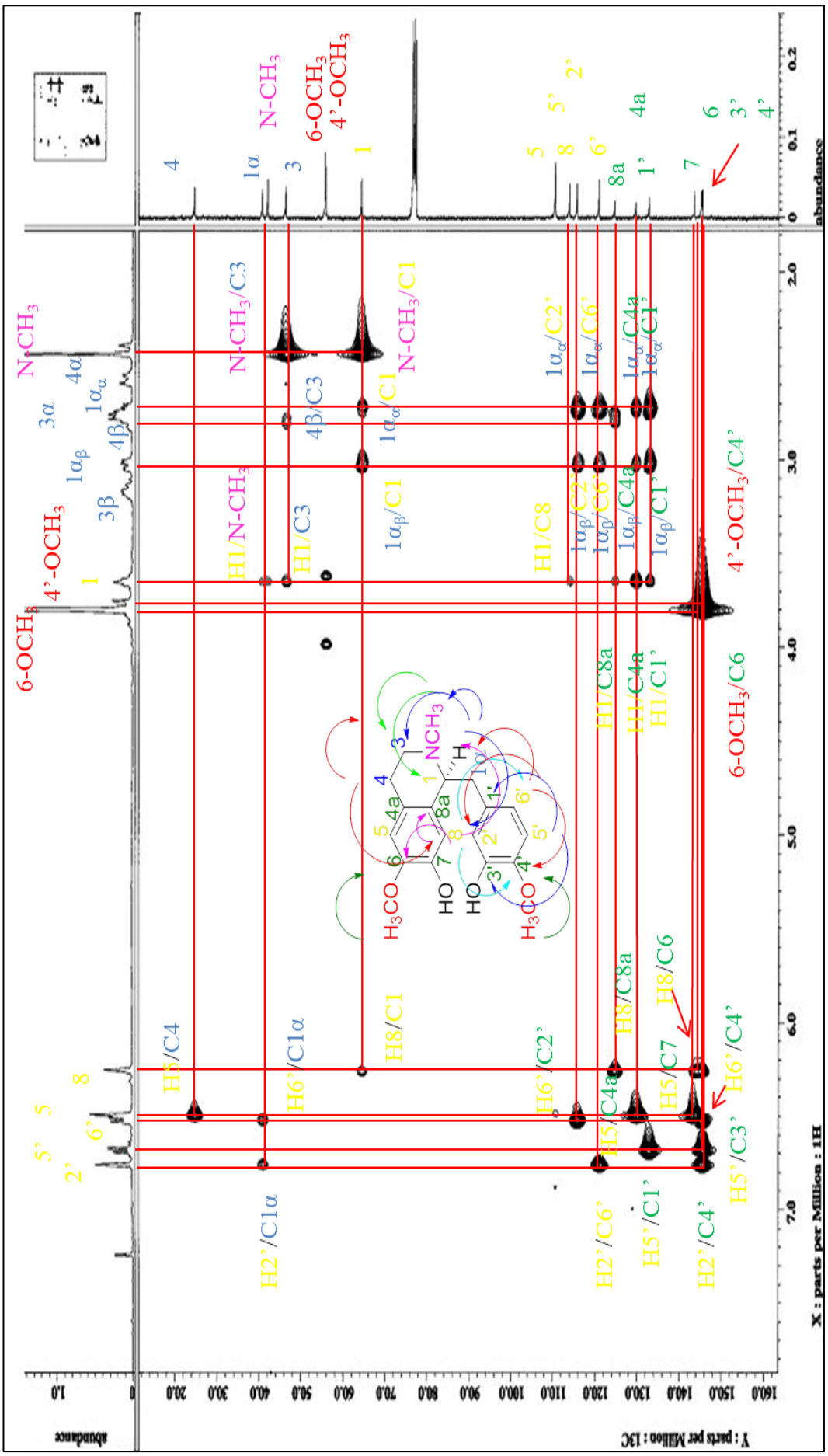
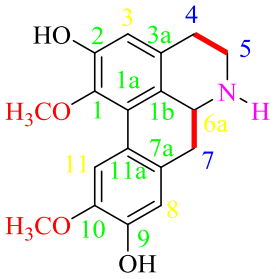


Figure 4.73: HMBC spectrum of reticuline (**61**)

4.3.6 Laurolitsine (6) or norboldine



6

Alkaloid **6** was isolated as a brownish amorphous solid. $[\alpha]_D^{25}$ = + 6.77° (c=0.5, MeOH). LCMS-Q TOF spectrum (Figure 4.74) revealed a pseudomolecular ion peak, $[M+H]^+$ at m/z 314.1397 corresponding to the molecular formula of $C_{18}H_{19}NO_4$ (calcd. for $C_{18}H_{20}NO_4$, 314.1314). Its UV spectrum showed absorption band at 282 nm and 307 nm. The degree of the resonance in the biphenyl system that existed in ring A and D, suggesting a 1,2,9,10-tetrasubstituted aporphine skeleton (Goodwin, *et al.*, 1958). In addition, the IR spectrum (Figure 4.75) gave a broad band between 3500 and 2500 cm^{-1} due to the presence of OH and NH functional groups. Its IR spectrum also showed a strong absorption at 2025 cm^{-1} due to the stretching of C-H aromatic, respectively. The UV and IR spectra of alkaloid **6** were typical of an aporphine carrying two hydroxyl groups.

The ^{1}H-NMR (Figure 4.76) also showed the existence of two methoxyl by the occurrence of two singlets at δ 3.59 and 3.87. These methoxyl groups most probably attached to C-1 and C-10. In addition, a singlet corresponding to one proton was observed at δ 6.60 which may be ascribed to H-3. This observation also indicated that C-2 is substituted. The position of methoxyl group was also proven by NOE different experiment (Figure 4.77) which showed enhancement of the methoxyl singlet at δ 3.59 and 3.87 when irradiated at H-11, thus confirming the position of OMe at C-1 and C-10. The respective positions of OMe at C-1 and C-10 were also comfirmed by the

experiment of NOESY (Figure 4.80). The aromatic ring D was substituted by hydroxyl and methoxy group at C-9 and C-10 respectively. Other peaks presence as a singlet signals at δ 6.73 and δ 7.95 were attributed to H-8 and H-11, respectively. These signals are typical of 1,2,9,10-tetrasubstituted aporphines (Shamma, 2012). H-11 is more deshielded due to the anisotropic effect caused by the ring A. The aliphatic protons of C-4, C-5, C-6a and C-7 resonated between δ 3.75 – 2.60. The cross correlation deduced from COSY spectrum (Figure 4.79) revealed the exact position of aliphatic protons H4/H5 and H6a/H7.

The ^{13}C-NMR spectrum (Figure 4.78) exhibited eighteen carbon resonances which closely resembled to those reported for laurolitsine (**6**) or norboldine. In addition, the signals are made up of two methoxyls, three methylenes, four methines and nine quaternary carbon signals. Four oxygenated aromatic quaternary carbon signals were observed at δ 149.5, 146.3, 142.3 and 146.8 which indicated the presence of hydroxyl at C2 and C9 and methoxyl group at C1 and C10, respectively. The methoxyl carbons were observed at δ 60.2 and δ 56.3 which further supported the structure of alkaloid **6**.

The HSQC spectra (Figure 4.81) support the full assignment of all the carbons and and aliphatic protons. HMBC spectra (Figure 4.82) confirmed the methoxyl position by cross peak correlation between 10-OCH_3/C10, H8/C10 for 10-methoxyl position. Hence, compound **6** is unambiguously assigned as laurolitsine (**6**) or norboldine in agreement with the previous reported data 1D-NMR (Guinaudeau *et al.,* 1975; Guinaudeau *et al.,* 1983; Guinaudeau *et al.,* 1994; Lee & Yang, 1992). The details NMR data of laurolitsine (**6**) is shown in the Table 4.12, since 2D-NMR (HMBC, HSQC and NOESY) data were not reported previously in the literature.

Table 4.12: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) data and 2D (HMBC and HSQC) NMR data of **6**, laurolitsine (**6**) or norboldine and the literature data.

	6 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^{2}J,^{3}J)	HSQC (^{1}J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	142.3	-	-	-	144.3
1a	-	126.5	-	-	-	127.5
1b	-	127.4	-	-	-	127.5
2	-	149.5	-	-	-	150.5
3	6.60 (*s*, 1H)	114.4	C_1,C_{1b}, C_2, C_4	H_3	6.63 (*s*, 1H)	115.6
3a		129.7	-	-	-	130.3
4	2.61 (*m*, 1H)	29.0	C_{1b}, C_3, C_{3a}, $C_{6a.}$	H_4		29.1
	2.91 (*m*, 1H)		C_{1b}, C_3, C_{3a}, $C_{6a.}$			
5	2.94 (*m*, 1H)	43.0	C_{3a},C_4,C_{6a}	H_5		43.8
	3.31 (*m*, 1H)		C_{3a},C_4,C_{6a}			
6a	3.75 (*dd*, *J*=13.7,4.1)	53.6	C_{1b},C_7,C_{7a}	H_{6a}		54.8
7	2.70 - 2.60 (*m*, 2H)	36.4	C_{1b},C_{6a},C_8,C_{11a}	H_7		37.0
7a	-	130.4	-	-	-	130.7
8	6.73 (*s*, 1H)	114.5	C_7,C_{10}, C_{11a}	H_8	6.73 (*s*, 1H)	115.9
9	-	146.3	-	-	-	147.1
10	-	146.8	-	-	-	147.7
11	7.95 (*s*, 1H)	110.6	C_{1a}, C_7, C_{7a}, C_8,C_9	H_{11}	7.97 (*s*, 1H)	113.0
11a	-	123.8	-	-	-	124.8
1-OCH_3	3.59 (*s*, 3H)	60.2	C_1	$3H_{1\text{-OMe}}$	3.59 (*s*, 3H)	60.3
10-OCH_3	3.87 (*s*, 3H)	56.3	C_{10}	$3H_{10\text{-OMe}}$	3.89 (*s*, 3H)	56.7

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Lee & Yang, 1992).

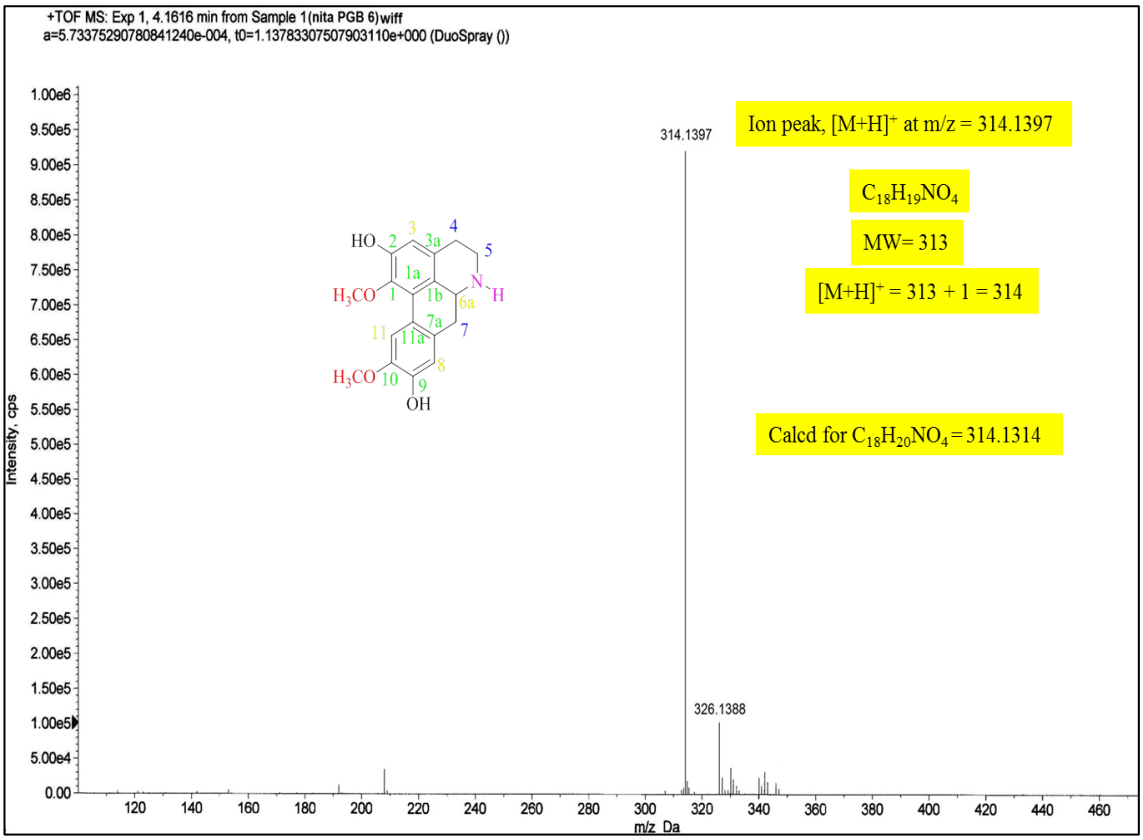


Figure 4.74: LCMS- Q TOF spectrum of **6**, laurolitsine (**6**).

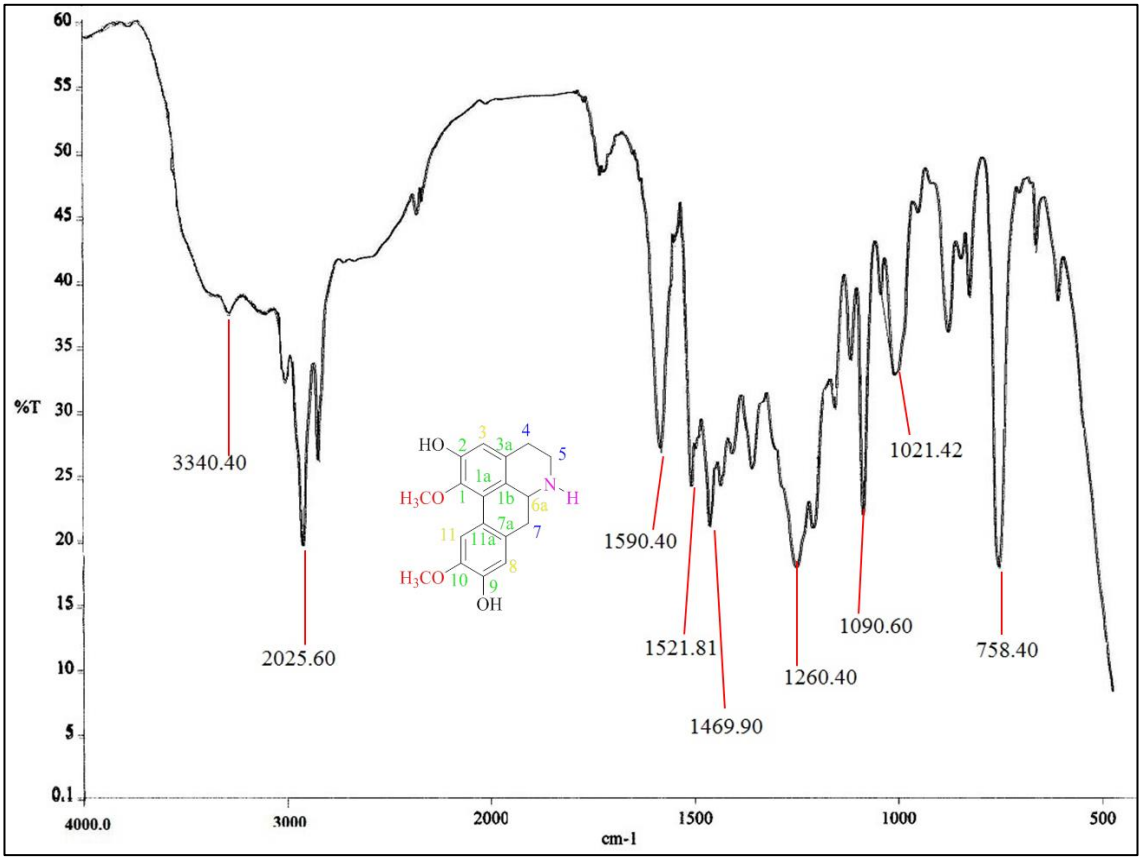


Figure 4.75: IR spectrum of **6**, laurolitsine (**6**).

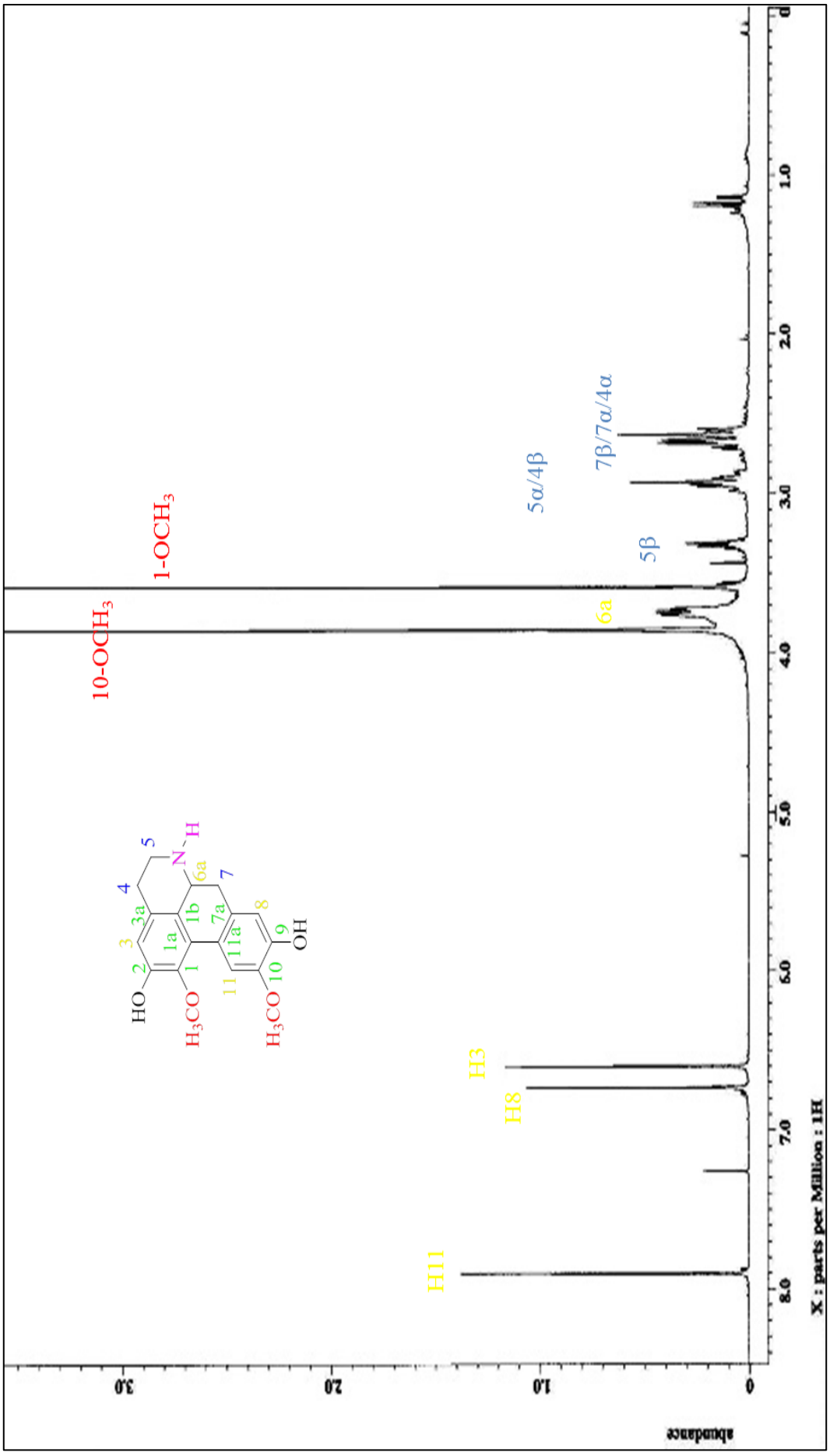


Figure 4.76: ^{1}H- NMR spectrum of **6**, laurolitsine (**6**)

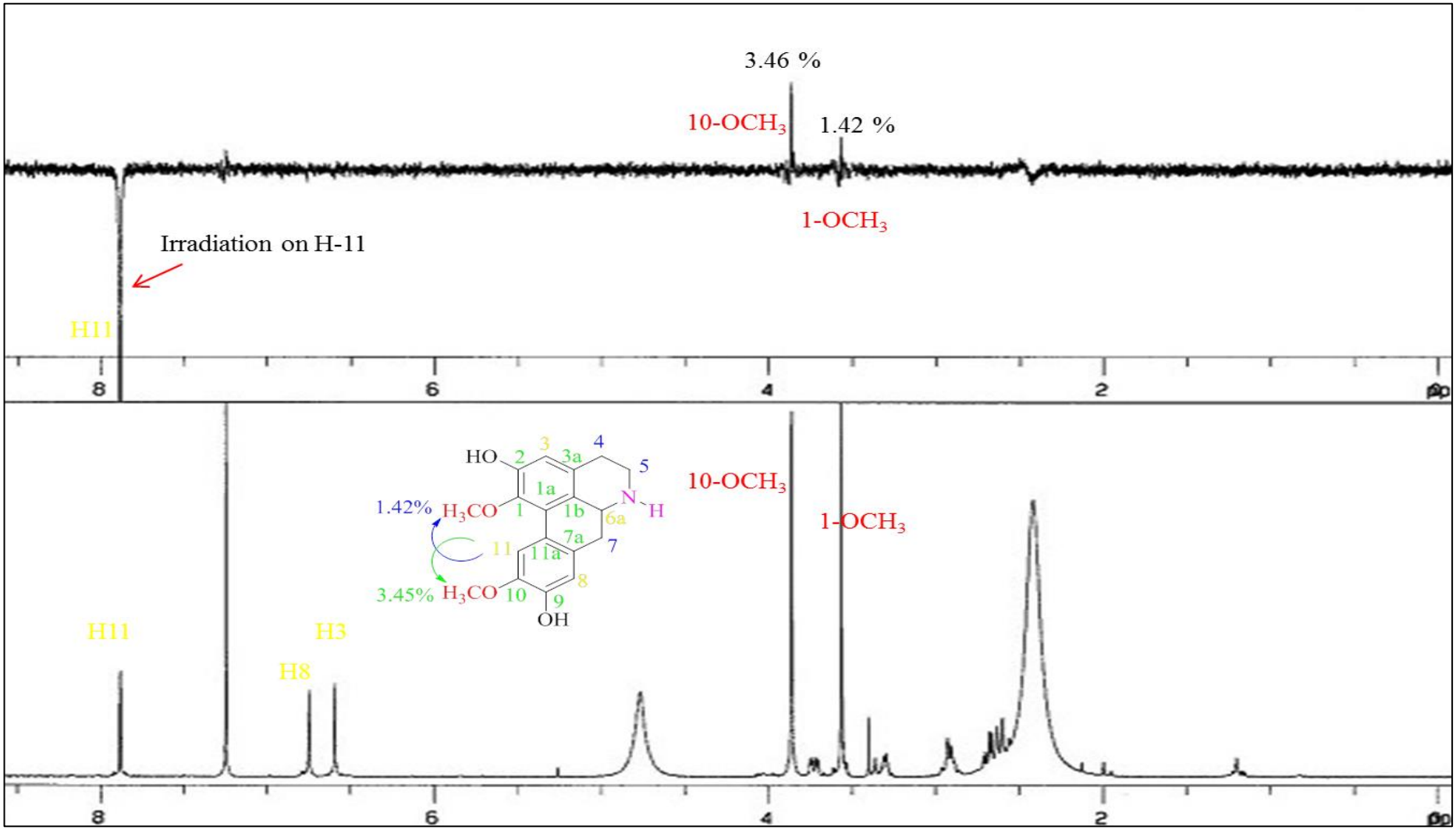


Figure 4.77: ^{1}H- NOE - Differential NMR spectrum of **6**, laurolitsine (**6**)

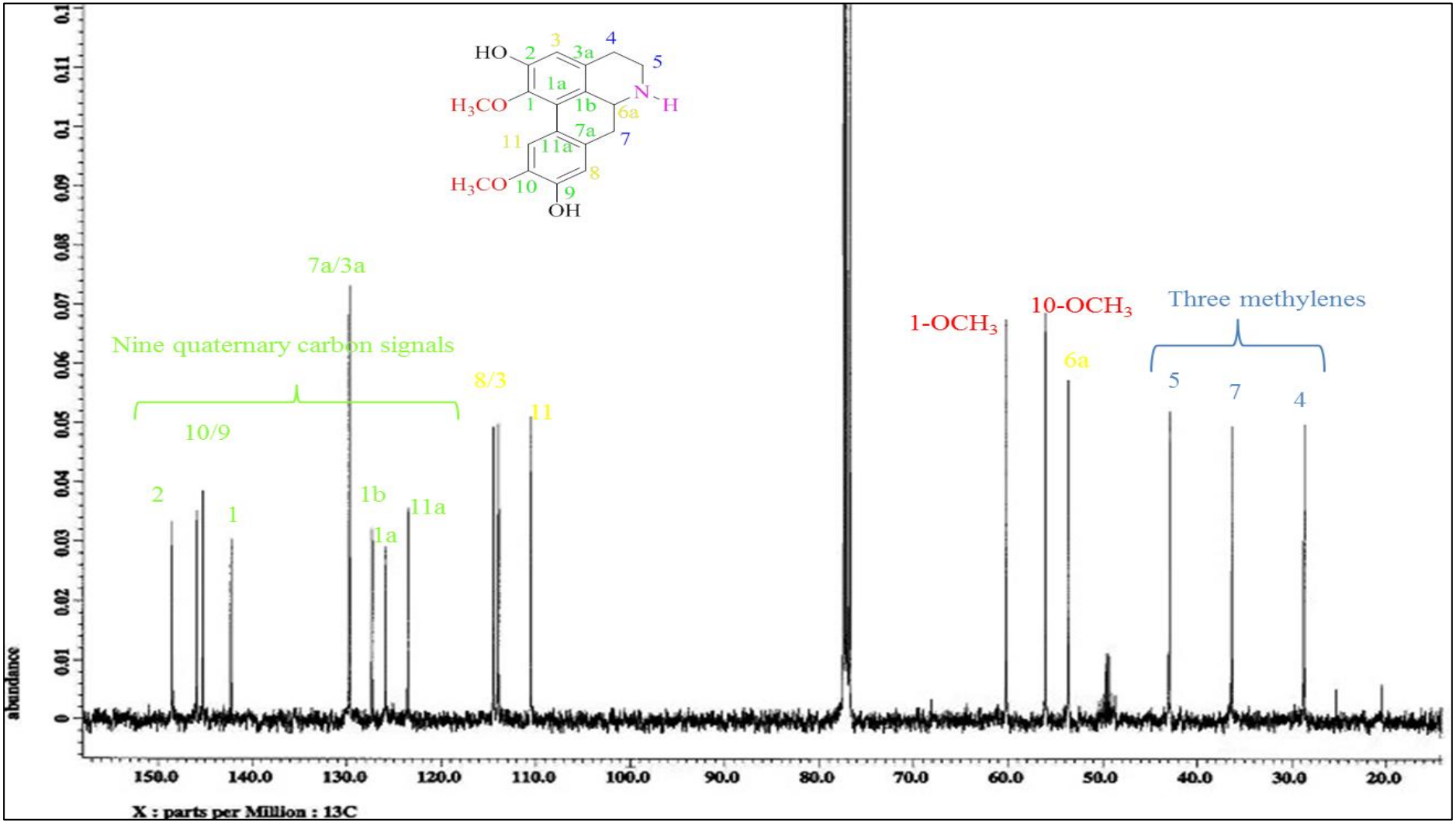


Figure 4.78: ^{13}C NMR spectrum of **6**, laurolitsine (**6**)

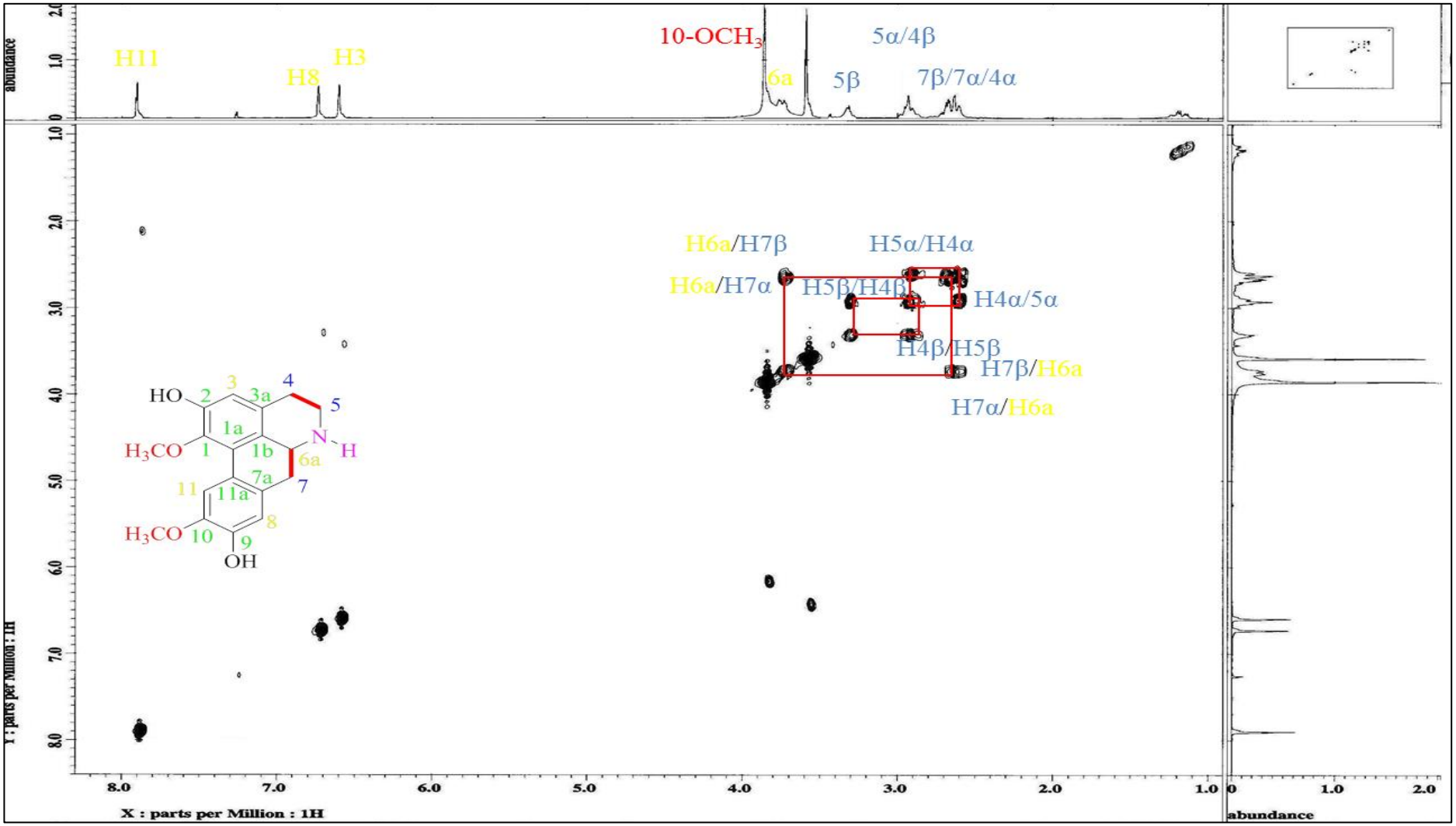


Figure 4.79: COSY spectrum of **6**, laurolitsine (**6**)

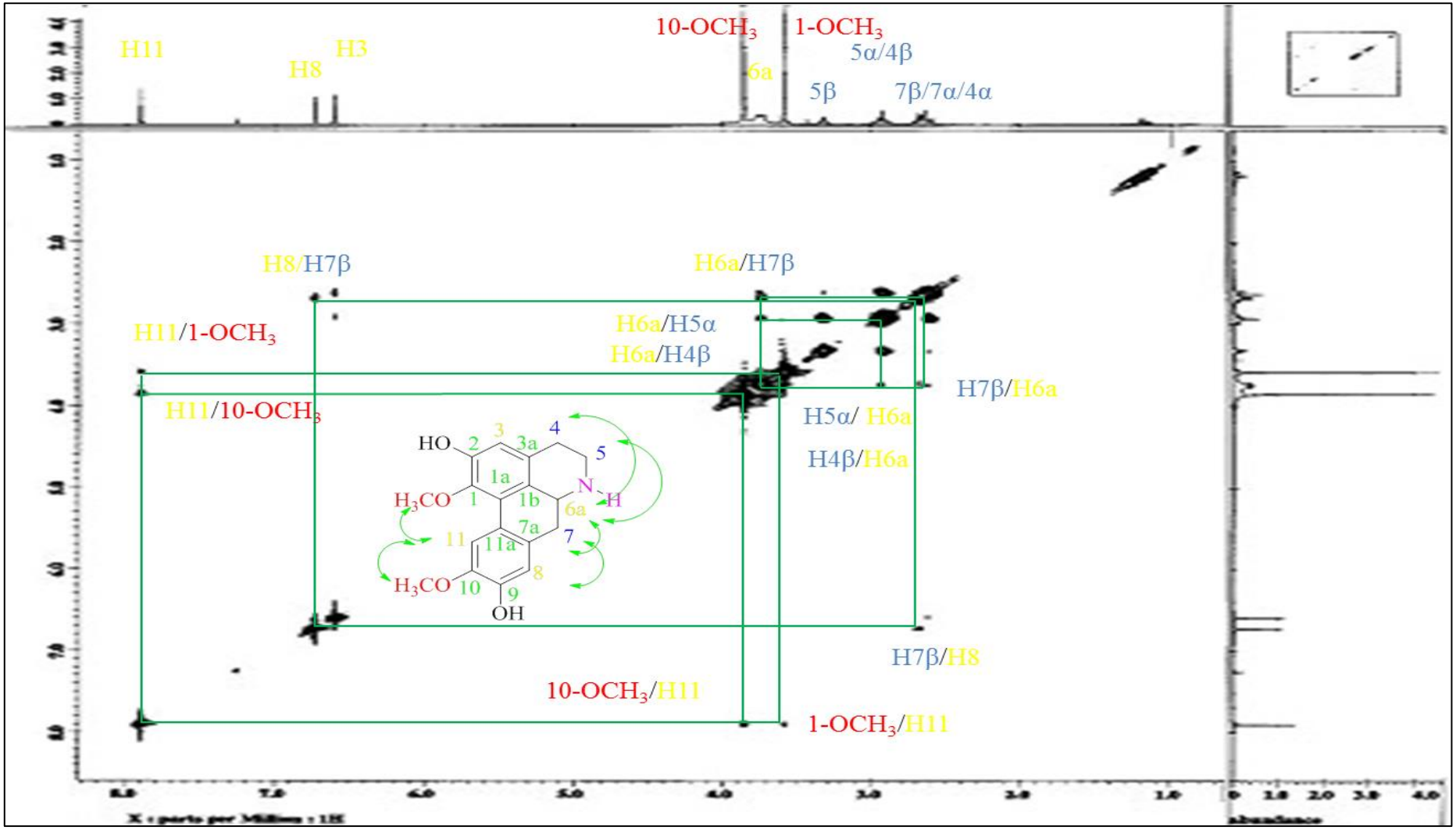


Figure 4.80: NOESY spectrum of **6**, laurolitsine (**6**)

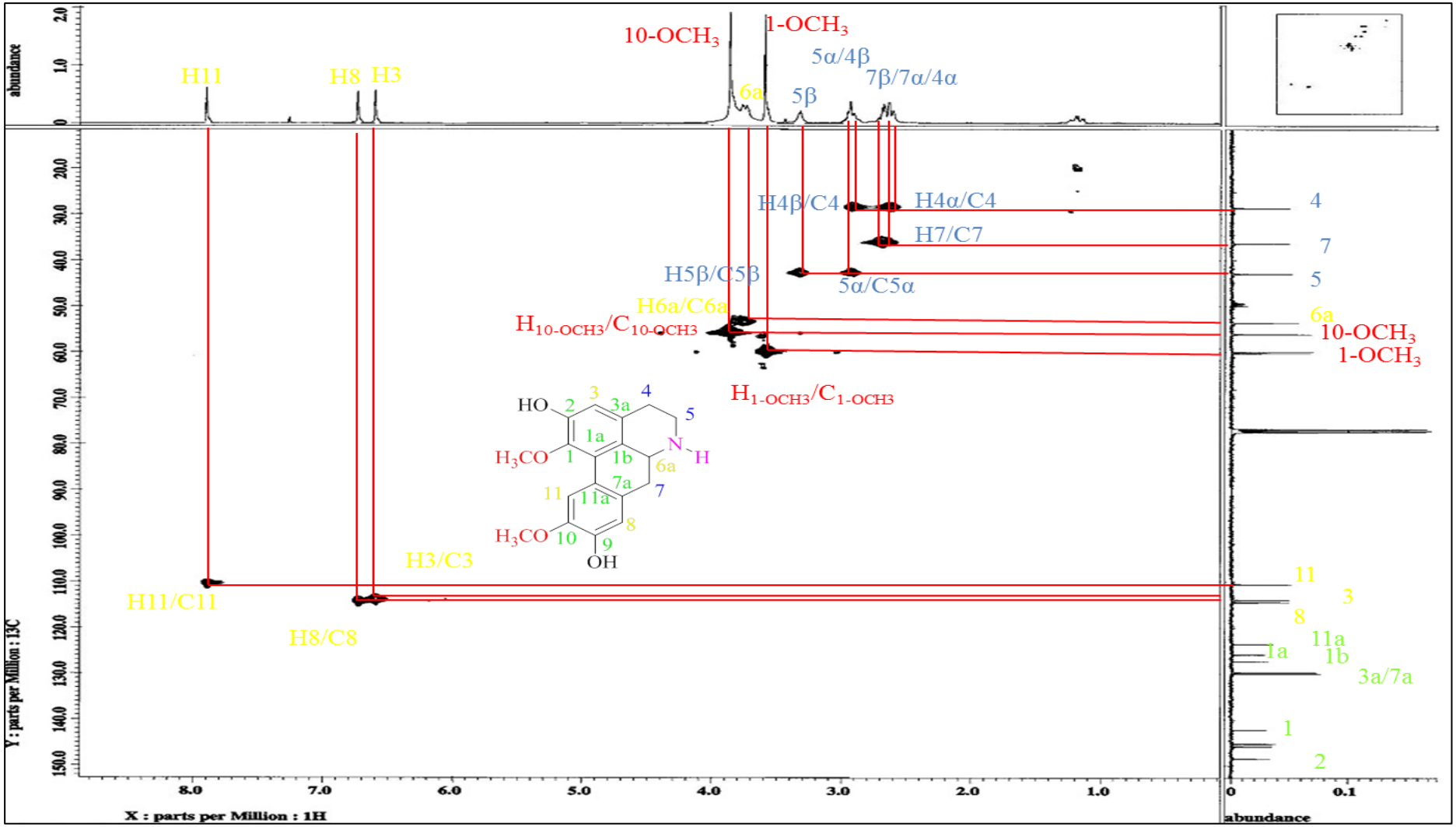


Figure 4.81: HSQC spectrum of **6**, laurolitsine (**6**)

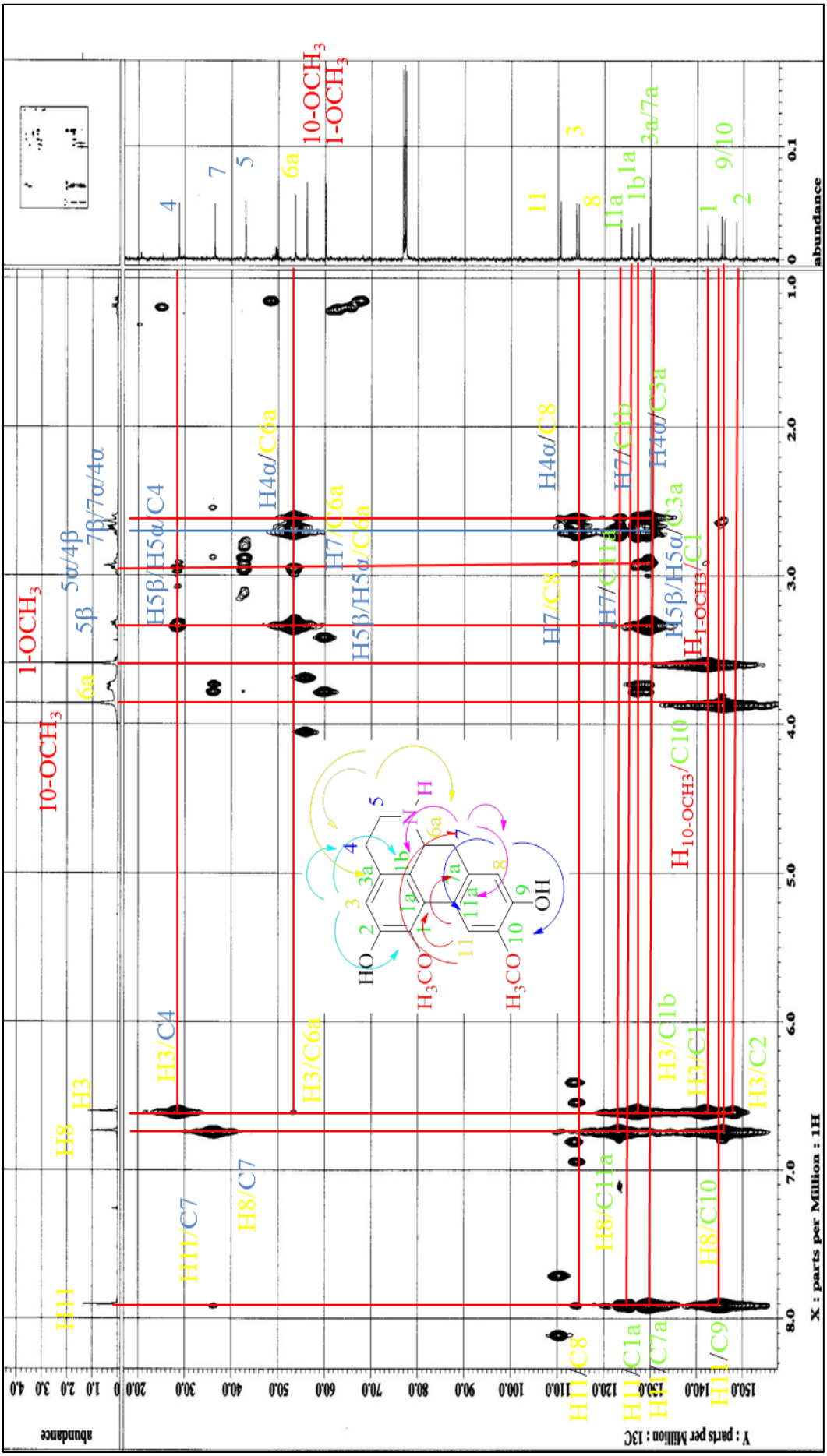
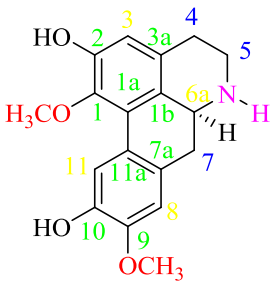


Figure 4.82: HMBC spectrum of **6**, laurolitsine (**6**)

4.4 Alkaloids isolated from the leaves of *Phoebe tavoyana* (KL 5225)

Further investigation on the phytochemistry of the leaves of *Phoebe tavoyana* species led to the isolation of new naturally occurring aporphine alkaloids; namely tavoyanine A (**63**) and tavoyanine B (**64**), together with four known aporphine alkaloids; laetanine (**62**), roemerine (**20**), laurolitsine **(6**), boldine (**5**) and besides one morphinandienones, sebiferine (**22**).

4.4.1 Laetanine (62)



62

Alkaloid **62** with $[\alpha]_D^{25}$ = + 105 (c=0.4, MeOH) with melting point 226-228°C was isolated as a dark brown amorphous solid. The LCMS-Q TOF spectrum (Figure 4.83) revealed an intense pseudomolecular ion peak, $[M+H]^+$ at m/z 314.1313 corresponding to the molecular formula of $C_{18}H_{19}NO_4$ (calcd. for $C_{18}H_{20}NO_4$, 314.1314). Its UV spectrum showed absorption band at 284 nm and 304 nm, a characteristic values for 1,2,9,10-tetrasubstituted aporphine (Guinaudeau *et al*., 1983). The IR spectrum (Figure 4.84) showed absorption peak range 3200 to 3400 cm^{-1} indicated the presence of hydroxyl and NH group in the structure (Guinaudeau *et al*., 1994).

The ^1H and ^{13}C NMR spectra data (Table 4.13) of compound **62** shared the same characteristics with that of compound **6** but the only difference was the substitution

pattern at ring D. The position C9 and C10 in **6** was substituted with hydroxyl and methoxyl group respectively whereas in **62** these positions were substituted with methoxyl and hydroxyl group respectively. Furthermore, the ^{1}H-NMR (Figure 4.85) also exhibited of two distinct methoxyl by revealing two singlets at δ 3.55 and 3.80 located at C-1 and C-9. Seven aliphatic protons were observed at high field region between δ 3.30 – 2.60 expected to occur at H-4, H-5, H-6a and H-7. A one proton singlet at δ 6.58 was assigned to H-3 confirming that C-1 and C-2 were substituted. Other peaks presence as singlets at δ 6.75 and δ 7.89 were attributed to H-8 and H-11, respectively. These values were typical of a 9,10 substitution pattern of aporphine moiety. H-11 is more deshielded due to the anisotropic effect caused by the ring A, and suggested that C-10 was substituted by a methoxyl group. The downfield shift of the C-11 and C-3 protons showed that the OH groups are at the C-2 and C-10 positions. The H-5 proton appeared at lower field compared to H-4 due to the neighbouring N-atom adjacent to C-5.

The ^{13}C-NMR experiments (Figure 4.86) established the resonance positions of eighteen carbons, which consist of three aromatic carbons, four aliphatic carbons, nine quaternary carbons and two methoxyl groups.

The ^{1}H-^{13}C direct correlations were determined by using HSQC spectrum (Figure 4.87) which showed the connectivity between proton and carbon; H3/C3, H4/C4, H7/C7, H6a/C6a, H5/C5, H8/C8, H9-OMe/C9-OMe, H1-OMe/C1-OMe and H11/C11.

The structure was finally confirmed by ^{1}H-^{13}C long range correlations in the HMBC spectrum (Figure 4.88). The exact positions of two methoxyls were confirmed by the correlations observed in the HMBC spectrum. The connectivities of both methoxyl

groups were determined by ^{3}J interaction between signals at δ 3.80 and quaternary aromatic carbon at δ 146.2 (C-9); and between signal at δ 3.55 and quaternary aromatic carbon at δ 142.7 (C-1) in the HMBC spectrum.

When compared with the spectroscopic data obtained for the compound **62** and literature report (Guinaudeau *et al*., 1983), it was confirmed that alkaloid **62** is indeed laetanine and have the structural formula as shown in Figure 4.83.

Table 4.13: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) data and 2D (HMBC and HSQC) NMR data of laetanine (**62**) and the literature data.

	62 in $CDCl_3$				* in $CDCl_3$
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)
1	-	142.7	-	-	-
1a	-	126.2	-	-	-
1b	-	124.9	-	-	-
2	-	149.1	-	-	-
3	6.58 (*s*, 1H)	113.9	C_1,C_{1b}, C_2, C_4	H_3	6.65 (*s*, 1H)
3a	-	128.4	-	-	-
4	2.98 (*m*, 1H)	27.4	C_{3a}	$H_{4\beta}$	
	2.66 (*m*, 1H)		C_{1b}, C_3, C_{3a}, $C_{6a.}$	$H_{4\alpha}$	
5	3.30 (*m*, 1H)	42.4	C_{3a},C_4,C_{6a}	$H_{5\beta}$	
	2.95 (*m*, 1H)		C_{3a},C_4,C_{6a}	H5α	
6a	3.80 (*dd*, *J*=14.2, 4.5)	53.4	C_{1b},C_{3a},C_{7a}	H_{6a}	4.15 (*dd*)
7	2.75 (*dd*, *J*=13.7, 4.5)	35.2	C_{1b}, C_{6a}, C7a, C_8	$H_{7\beta}$	
	2.60 (*dd*, *J*=13.7, 4.5)		C_{1b}, C_{6a}, C7a, C_8	H7α	
7a	-	128.6	-	-	-
8	6.75 (*s*, 1H)	114.5	C_7, C_{10}, C_{11a}	H_8	6.77 (*s*, 1H)
9	-	146.2	-	-	-
10	-	145.5	-	-	-
11	7.89 (*s*, 1H)	110.9	C_{7a}, $C_{9,}$ C_{11a}	H_{11}	7.91 (*s*, 1H)
11a	-	123.3	-	-	-
1-OCH_3	3.55 (*s*, 3H)	60.1	C_1	$3H_{1\text{-OMe}}$	3.60 (*s*, 3H)
9-OCH_3	3.80 (*s*, 3H)	56.1	$C_{9,}$ C_{10}	$3H_{9\text{-OMe}}$	3.80 (*s*, 3H)

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Guinaudeau *et al*., 1983).

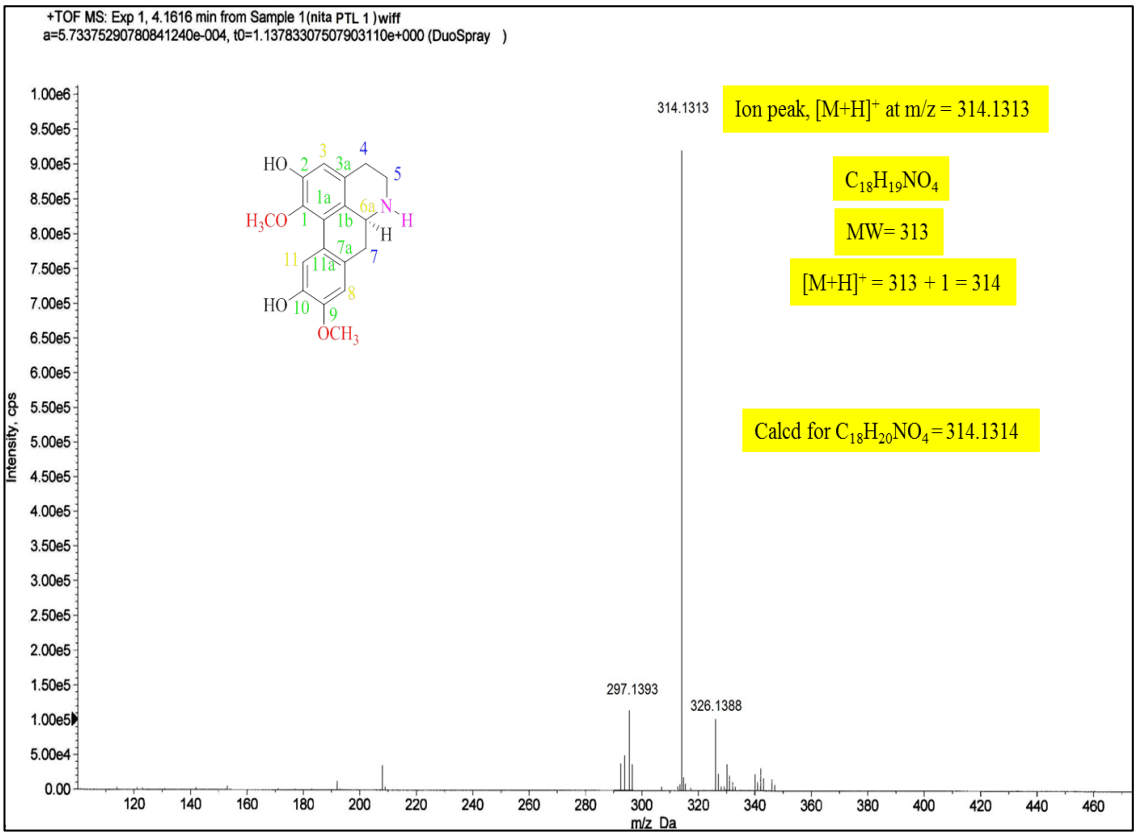


Figure 4.83: LCMS- Q TOF spectrum of laetanine (**62**).

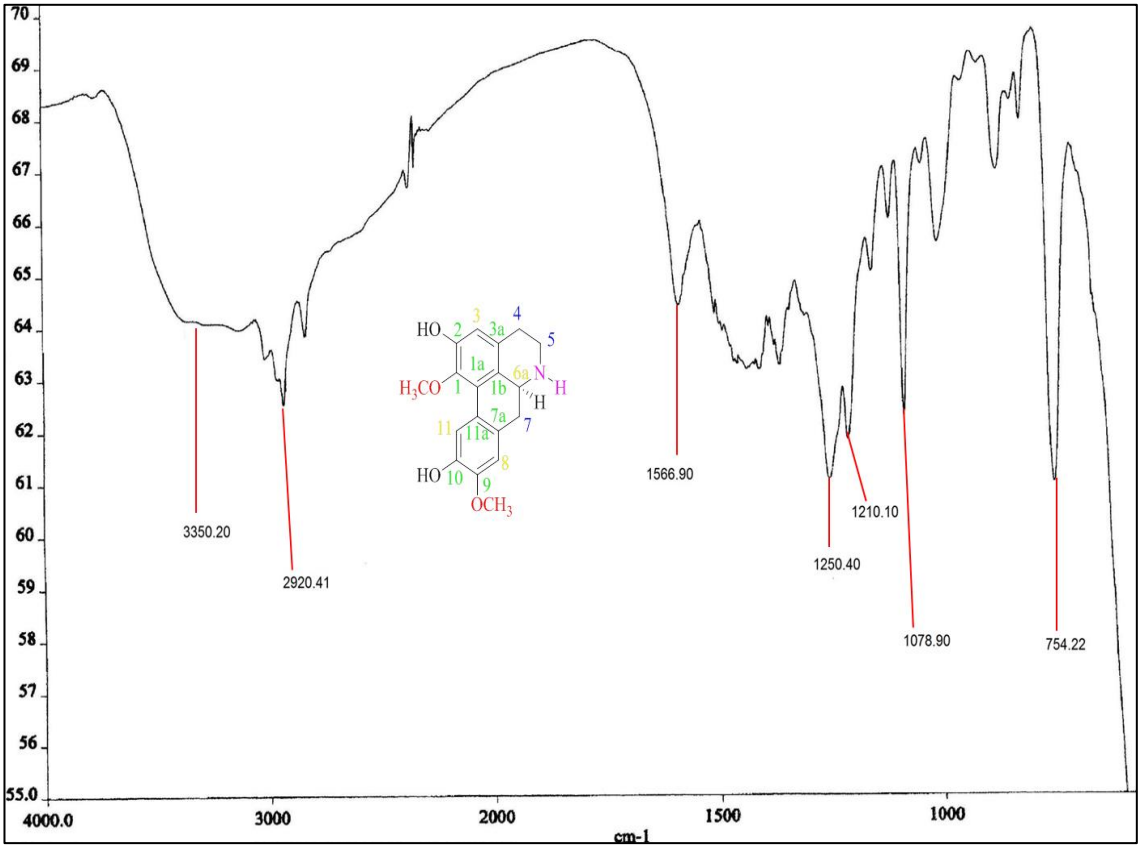


Figure 4.84: IR spectrum of laetanine (**62**).

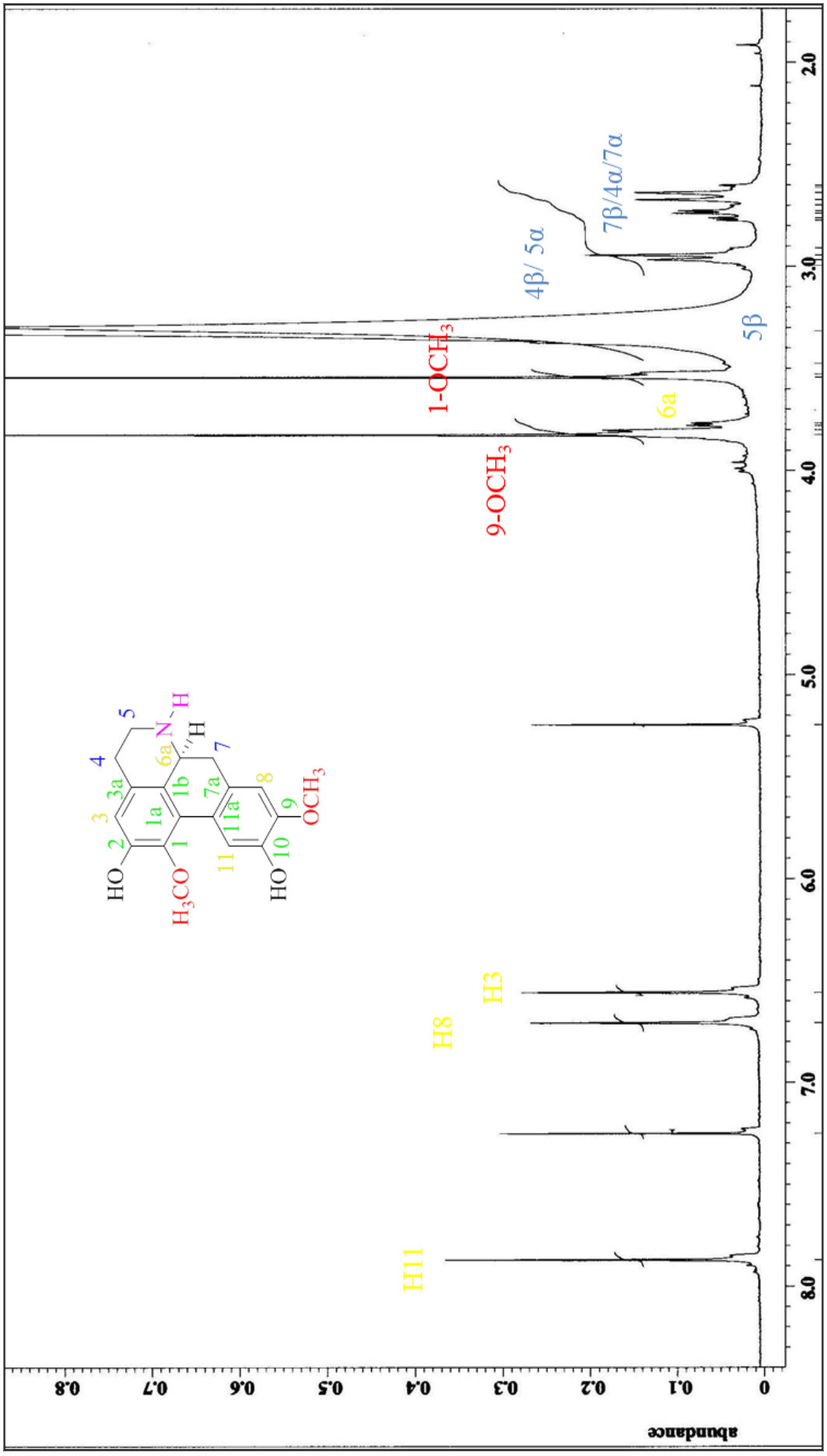


Figure 4.85: ^{1}H- NMR spectrum of laetanine (**62**)

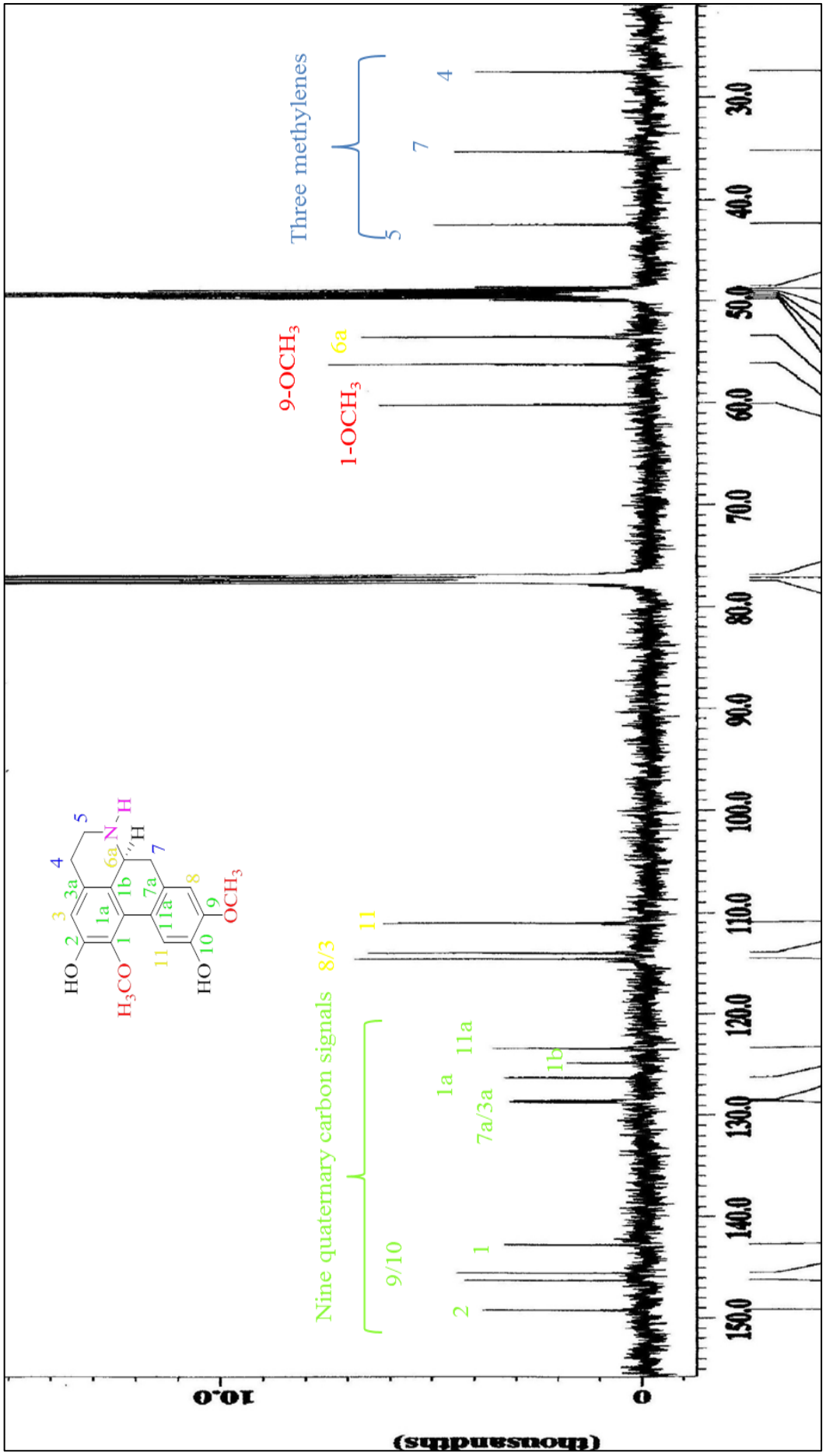


Figure 4.86: ^{13}C NMR spectrum of laetanine (**62**)

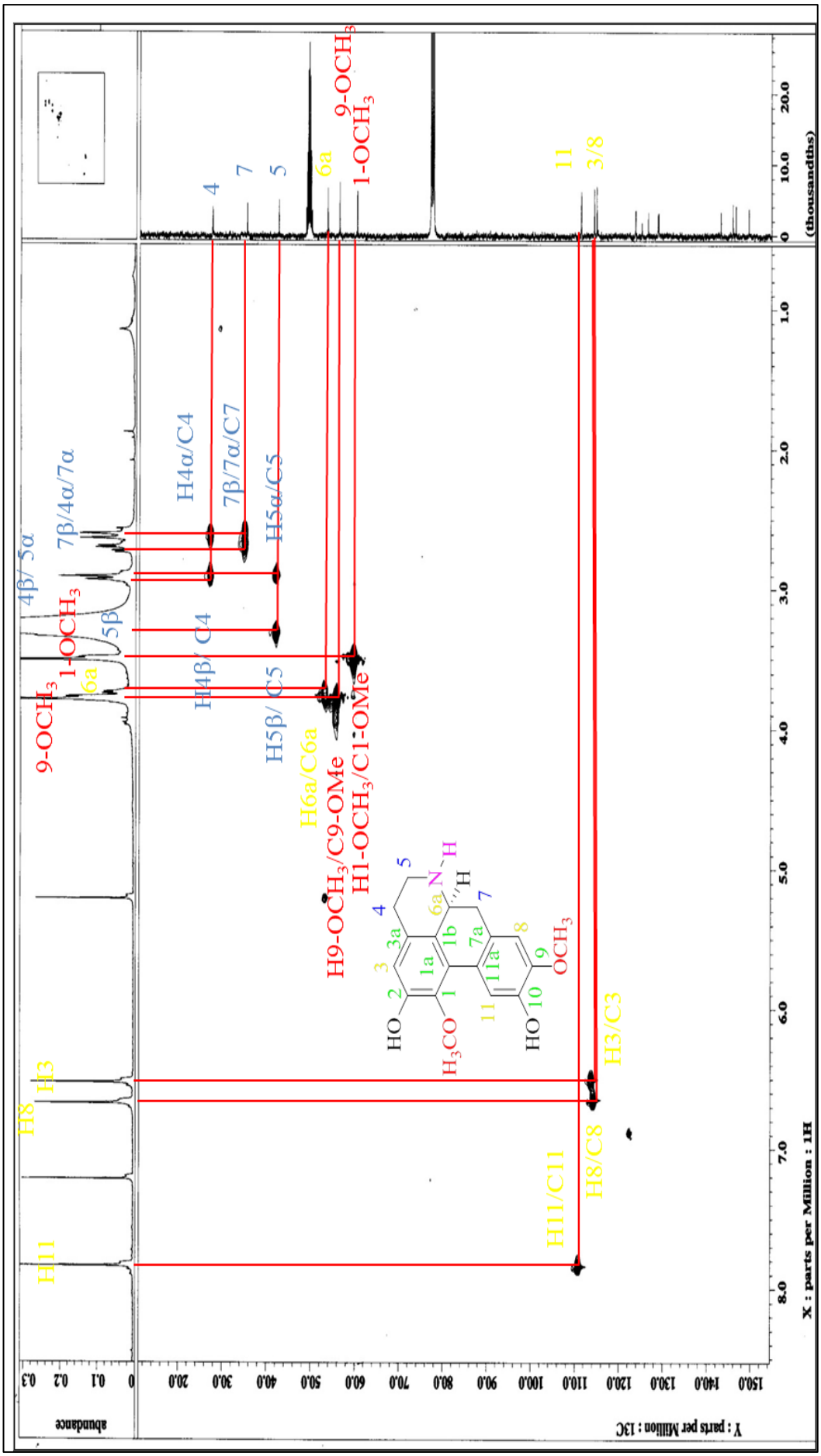


Figure 4.87: HSQC spectrum of laetanine (**62**)

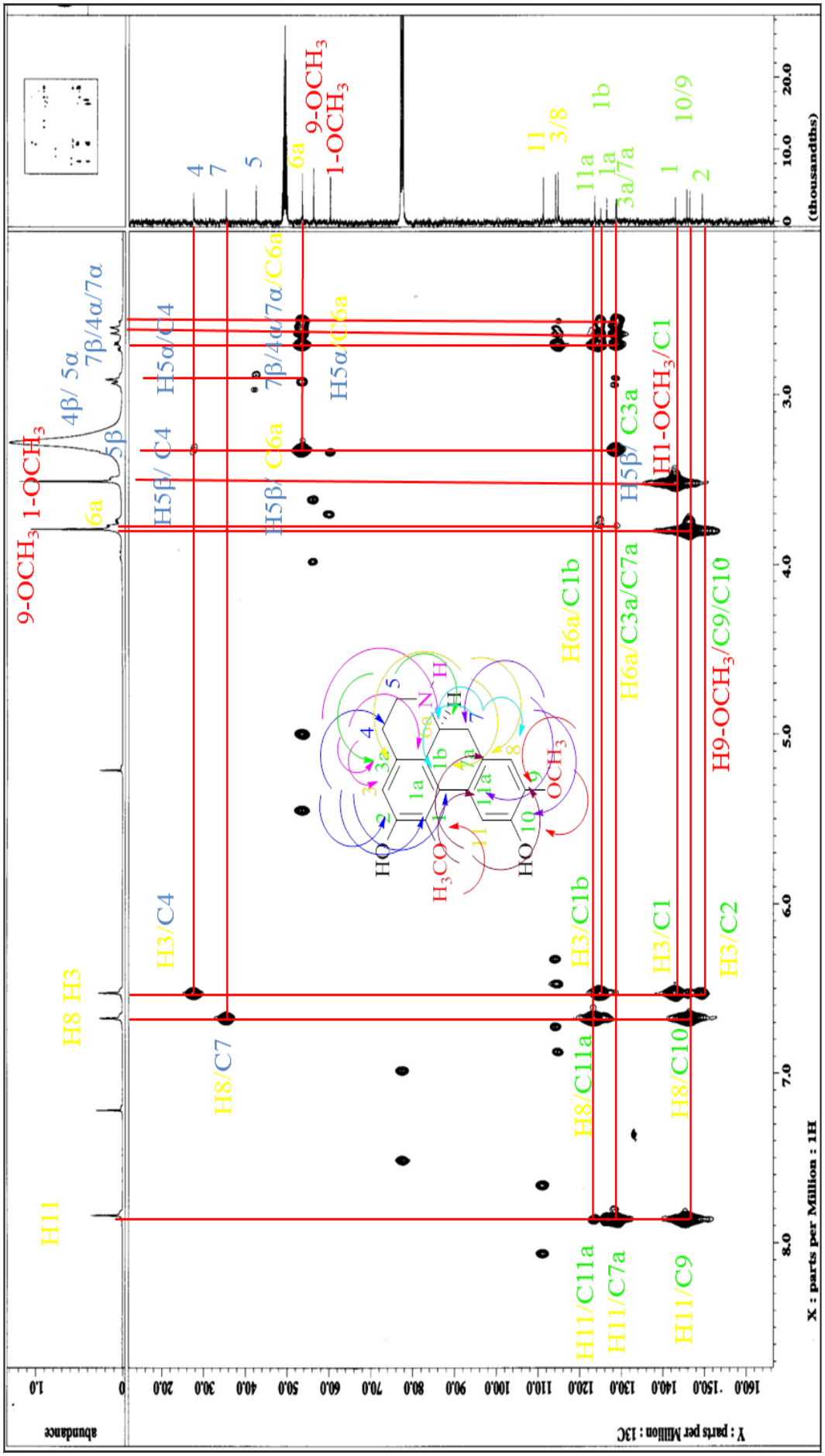
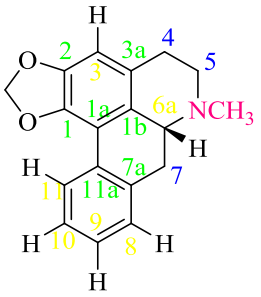


Figure 4.88: HMBC spectrum of laetanine (**62**)

4.4.2 Roemerine (20) or N-methylanonaine



20

Alkaloid **20** with $[\alpha]_D^{25}$ = - 0.180° (c= 0.5, C_2H_6OH) was isolated as white amorphous solid. The LCMS-Q TOF spectrum (Figure 4.89) revealed an intense pseudomolecular ion peak, $[M+H]^+$ at m/z 280.1257, which correlated to a molecular formula of $C_{18}H_{17}NO_2$ (calcd. for $C_{18}H_{18}NO_2$, 280.1259). Its UV spectrum showed maxima absorption band at 234, 264, 273, 285, 293 and 320 nm, thus suggesting a 1, 2 disubstituted aporphine skeleton (Guinaudeau *et al*., 1983). The IR spectrum (Figure 4.90) exhibited absorption band at 1039 cm^{-1} which was characteristic of the C-O stretching vibrations of methoxyl or methylenedioxy group. A peak at 953 cm^{-1} was also observed which was assignable to a methylenedioxy group (Chang *et al*., 2000), (Doskotch *et al*., 1969). Moreover, bands for C=C aromatic were also observed at 1450 and 1410 cm^{-1}.

An integration of the ^1H NMR spectrum (Figure 4.91) exhibited signals for five aromatic protons. A low field signal, observed as a doublet at δ 8.04 (*J*=7.0 Hz) assigned to H-11 due to the deshielding effect of the facing aromatic ring A. A set of multiplets centered at δ 7.20 – 7.32 was observed too, which was attributed to three aromatic protons, H-8, H-9 and H-10 on ring D. Therefore, ring D was free from any substituent. A singlet corresponding to one proton was observed at δ 6.55 which may be ascribed to H-3. In addition signals due to methylenedioxy protons were observed as a

doublet at δ 5.93 and 6.08 ppm (2H, *d*, *J*= 1.4 Hz, -OCH_2O-) and *N*-methyl group at δ 2.67 ppm.

The assignment of the aromatic protons on ring D was also confirmed by analysis of the COSY data. In the COSY spectrum (Figure 4.93), H11 is coupled to H10, H6a is coupled to H7 and H5 is coupled to H4. These match with the suggested structure.

The ^{13} C-NMR spectrum (Figure 4.92) gave a total of eighteen carbons which validated the molecular formula $C_{18}H_{17}NO_2$. The ^{13} C-NMR experiment also confirmed the presence of five methines, seven quaternary carbons, N-Me atom at δ 43.6 and one methylenedioxy group at δ 100.6 in the structure. Other chemical shift values of NMR data of alkaloid **20** were given in the Table 4.14.

Based on these data, the structure for alkaloid **20** is assigned as roemerine or *N*-methyl-anonaine. The spectral data were in full agreement with reported values (Chang *et al*., 2000; Mukhtar, 2003) of roemerine **(20)** or *N*-methyl-anonaine.

Table 4.14: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of roemerine **(20)** and the literature data.

	20 in $CDCl_3$		* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	142.5	-	142.3
1a	-	116.3	-	116.1
1b	-	126.3	-	126.2
2	-	146.6	-	146.4
3	6.55 (*s*, 1H)	107.3	6.55 (*s*, 1H)	107.4
3a	-	128.1	-	127.8
4	2.90 (*d*, *J*=10.2) 2.70 (*d*, *J*=10.2)	28.9	2.98 m 2.68 m	28.8
5	3.24 (*d*, *J*=4.2) 3.19 (*d*, *J*=4.4)	53.4	2.98 – 3.34 m	53.2
6a	3.49 (*d*, *J*=13.7)	61.9	3.78 d (12.5)	61.8
7	2.73 *m* 2.71 *m*	34.3	2.72 m 2.68 m	34.3
7a	-	135.1	-	135.1
8	7.20 - 7.32 *m*	126.5	7.27 m	126.5
9	7.20 - 7.32 *m*	126.7	7.27 m	126.7
10	7.20 - 7.32 *m*	126.9	7.27 m	126.8
11	8.04 (*d*, *J*=7.0)	127.4	8.04 *d*	127.2
11a	-	130.9	-	130.8
OCH_2O	5.93 *d* (*J*=1.4) 6.08 *d* (*J*=1.4)	100.6	5.92 6.06	100.4
N-Me	2.67 (*s*, 3H)	43.6	2.53 (*s*, 3H)	43.5

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Mukhtar, 2003).

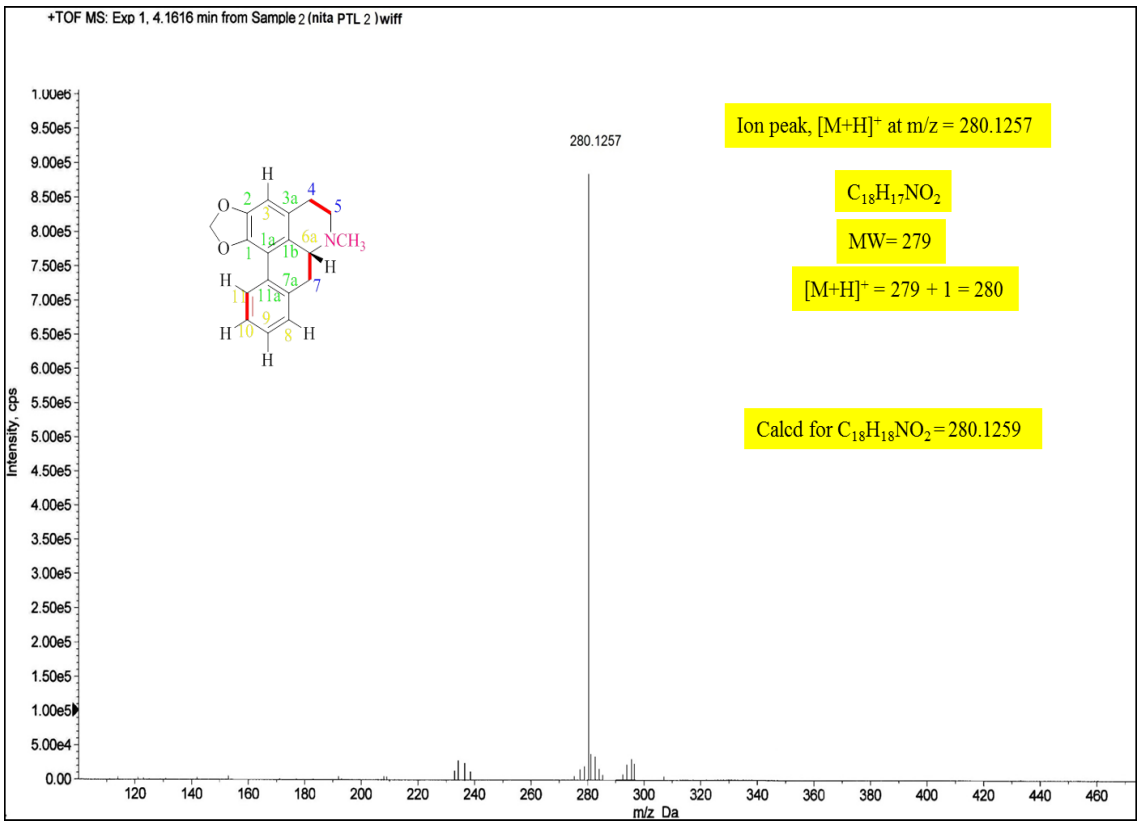


Figure 4.89: LCMS- Q TOF spectrum of roemerine (**20**) or N-methylanonaine.

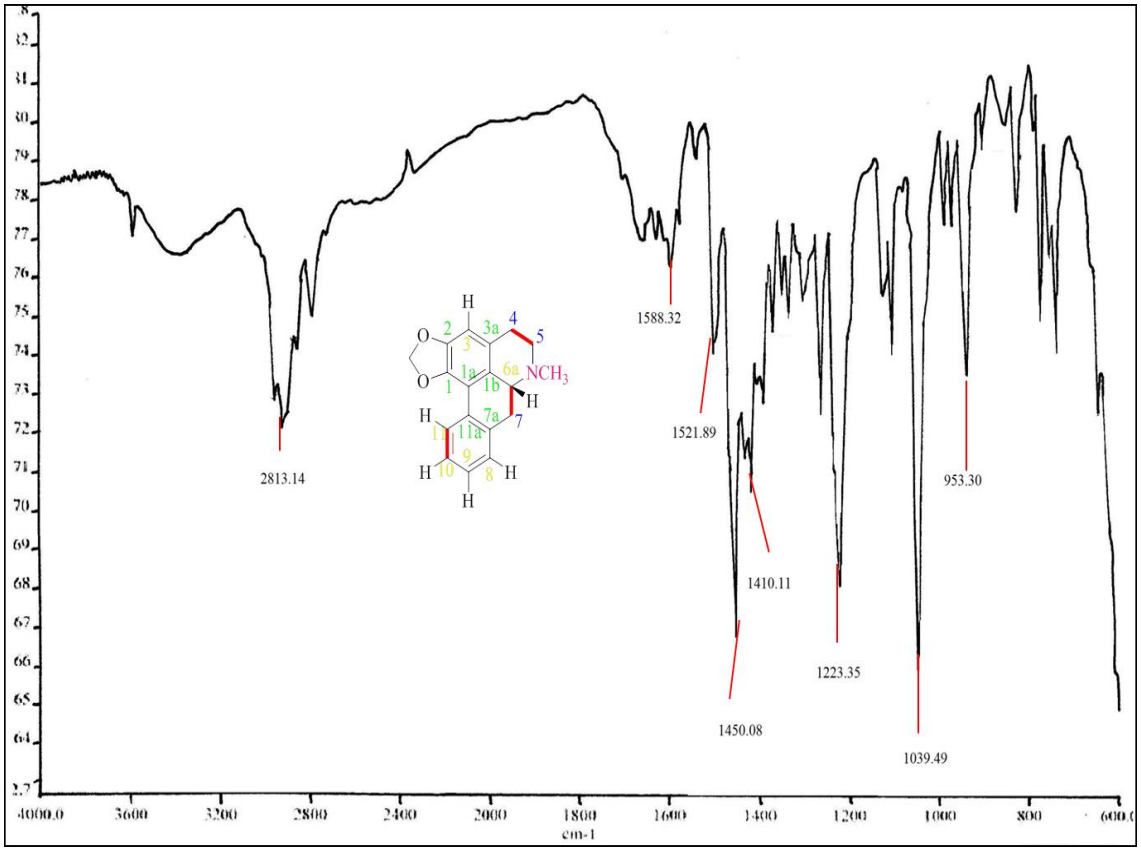


Figure 4.90: IR spectrum of roemerine (**20**) or N-methylanonaine.

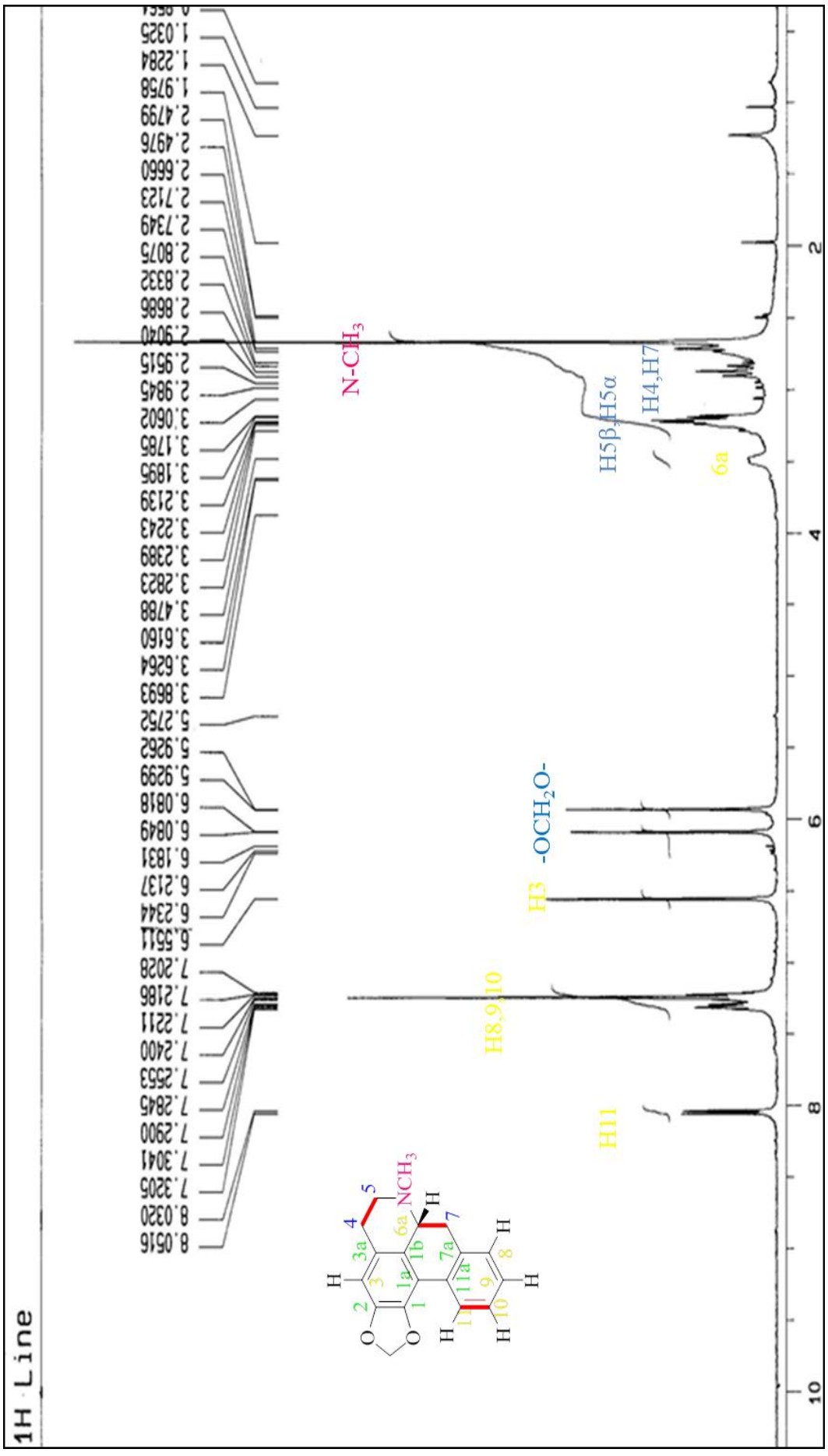


Figure 4.91: ^{1}H- NMR spectrum of roemerine (**20**) or N-methylanonaine

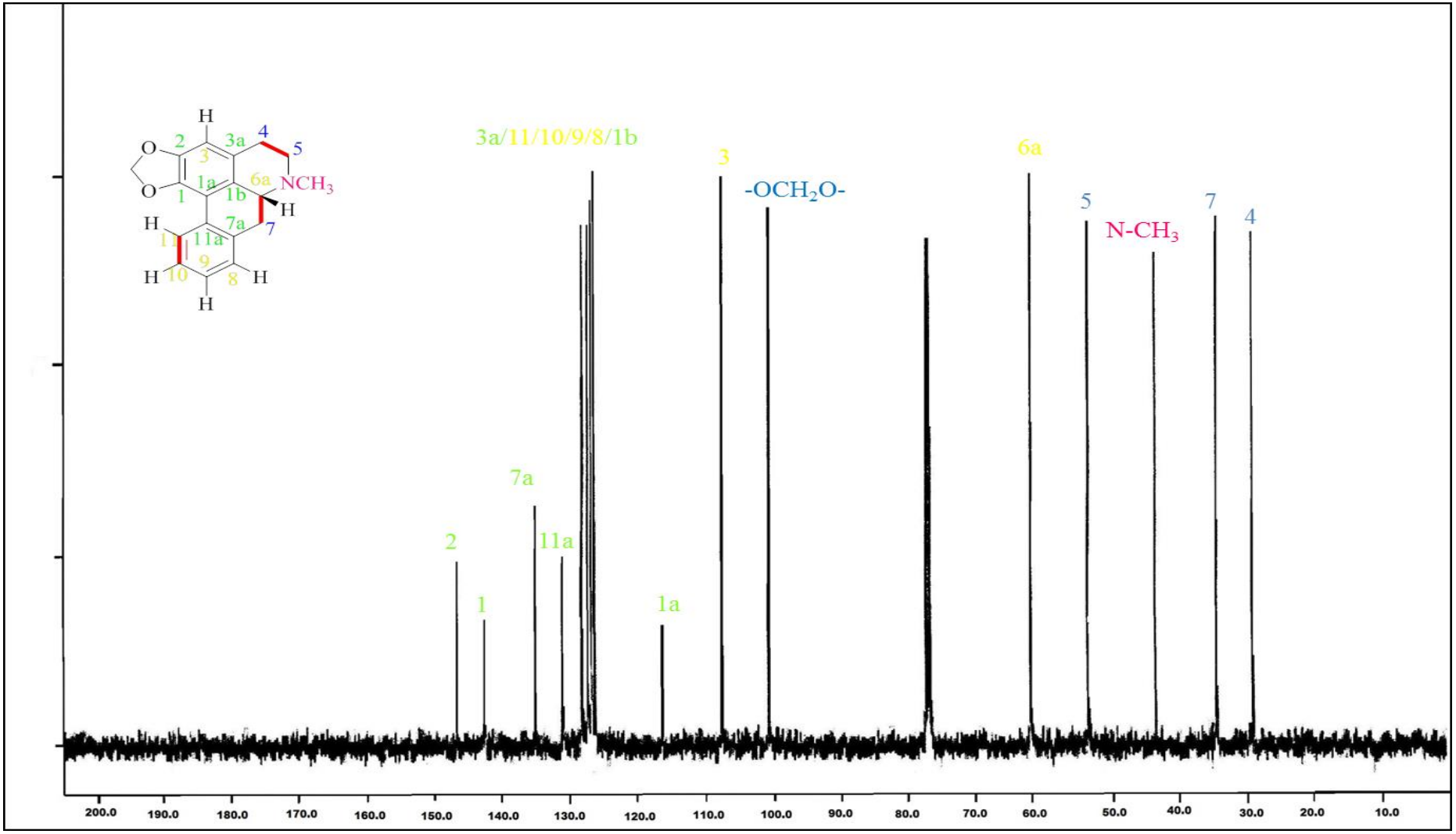


Figure 4.92: ^{13}C NMR spectrum of roemerine (**20**) or N-methylanonaine

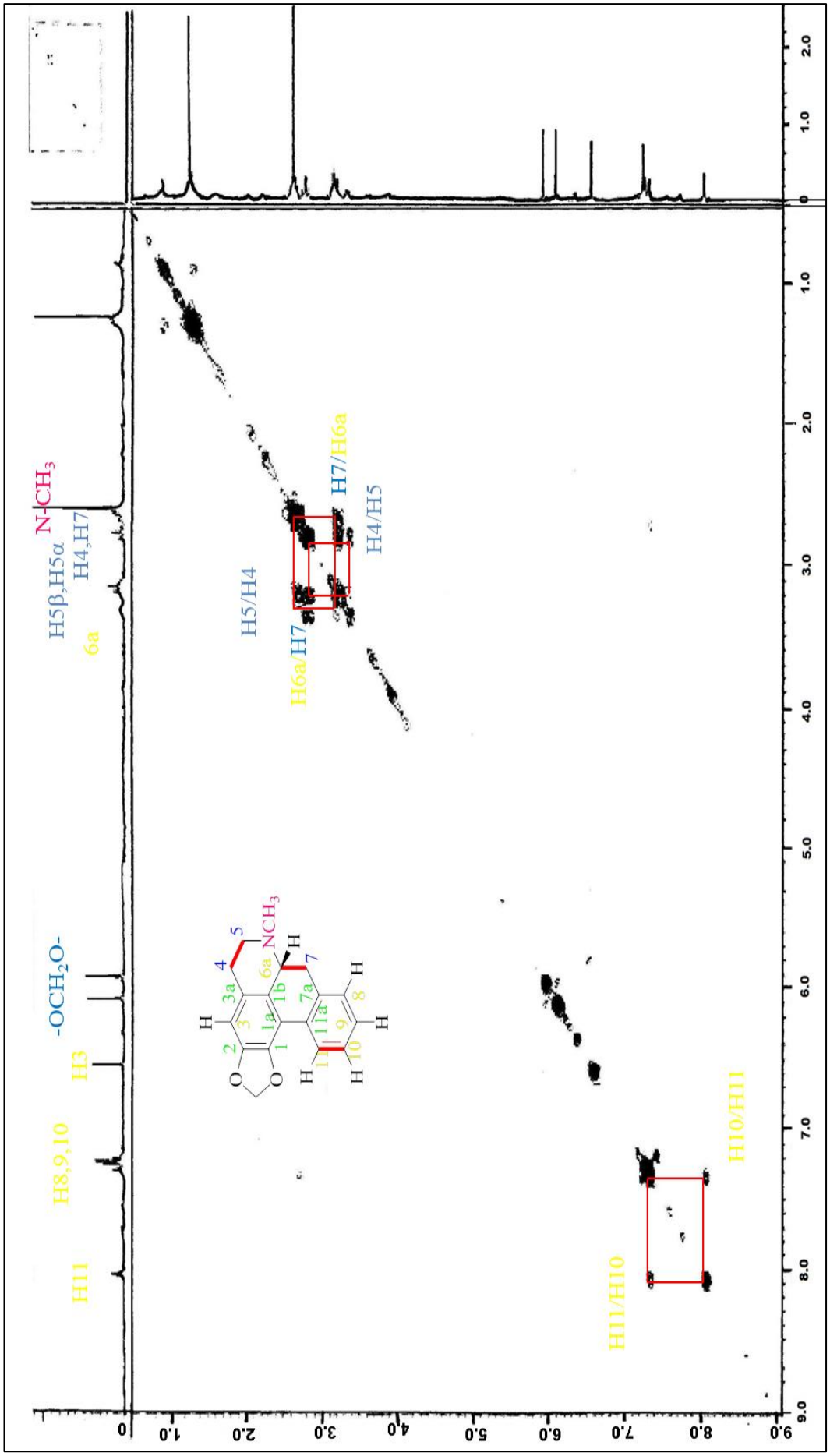
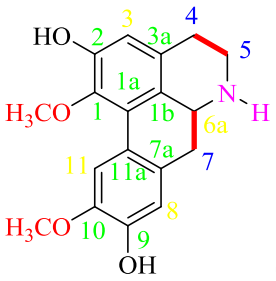


Figure 4.93: COSY spectrum of roemerine (**20**) or N-methylanonaine.

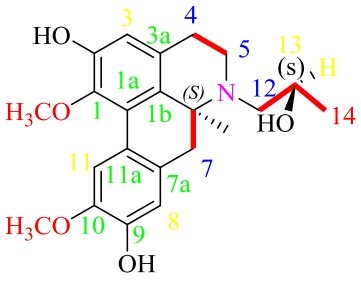
4.4.3 Laurolitsine (6)



6

Alkaloid **6** was isolated as a brownish amorphous solid. $[\alpha]_D^{25}$ = + 6.77° (c=0.5, MeOH). Its UV spectrum showed absorption band at 282 nm and 307 nm. The degree of the resonance in the biphenyl system that existed in ring A and D, suggesting a 1,2,9,10-tetrasubstituted aporphine skeleton (Goodwin *et al*., 1958). In addition, the IR spectrum gave a broad band between 3500 and 2500 cm^{-1} due to the presence of OH and NH functional groups. The MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HMQC and NOESY) data spectra features were similar to those of previous isolated laurolitsine (**6**) or norboldine. Please refer to **6** (page 203-213).

4.4.4 (+)-Tavoyanine A (63)



63

Alkaloid **63** was obtained as a dark brown amorphous solid. $[\alpha]_D^{25}$ = + 0.016° (c= 0.5, CH_3OH). The LCMS-ESI electrospray ionisation mass spectrometry (Figure 4.94), exhibited an ion peak at *m/z* 372.1776 $[M+H]^+$ giving a possible molecular formula of $C_{21}H_{25}NO_5$, with the *m/z* (calcd. for $C_{21}H_{26}NO_5$, 372.1811). Its UV spectrum showed

absorption band at 283 nm and 305 nm, thus suggesting a 1,2,9,10-tetrasubstituted aporphine skeleton (Goodwin *et al*., 1958). In addition, a broad band at 3360 cm^{-1} in the IR spectrum (Figure 4.95) indicated the presence of phenolic hydroxyl moiety. Its IR spectrum also showed strong absorptions at 2928 cm^{-1} and 2825 cm^{-1} due to the stretching of C-H aromatic, respectively. The absorptions from 1465 – 1510 cm^{-1} indicated aromatic C=C stretching. The strong absorption at 1258 cm^{-1} indicated the presence of the methoxyl groups. Moreover, the UV and IR spectra of alkaloid **63** were typical of an aporphine carrying two hydroxyl groups.

The ^{1}H NMR spectrum (Figure 4.96) of **63** revealed two aromatic methoxyl singlets at δ 3.85 and δ 3.50 attached to C10 and C1, respectively. Three ^{1}H singlets were observed at δ 7.80, δ 6.76 and δ 6.58. The former signal was assigned to H-11 and the latter was assigned to H-8 on ring D and H-3 on ring A. H-11 showed the highest chemical shift due to the deshielding effect of the ring A. The spectrum also revealed the presence of -$CH_2CHOHCH_3$ group attached to the nitrogen atom with the occurrence of a doublet at δ 1.12 (*J*=6.4 Hz) due to the presence of methyl group (C-14) next to a methine proton (C-13). In thru this proton resonance at δ 3.88 as multiplet since it also coupled to the methylene proton at H-12 which occurred at δ 2.70 (*dd, J*=2.7, 12.6 Hz) and δ 2.18 (*dd, J*=2.7, 12.6 Hz) were attributed to H12β and H12α, respectively. Furthermore, four aliphatic protons were observed at δ 3.11 (*dd, J*=4.1, 11.3 Hz), δ 2.60 (*d, J*=4.3 Hz), δ 2.56 (*d, J*=4.3 Hz) and δ 2.38 (*dd, J*=4.1, 11.3 Hz) attributable to H5β, H4β, H4α and H5α, respectively. The signal for H7β and H7α appeared as doublet of doublet at δ 2.94 (*dd, J*=3.7, 12.0 Hz) and δ 2.42 (*dd, J*=3.7, 12.0 Hz). Finally, the remaining signals of one proton as a pair of doublet, (*dd, J*=3.2, 13.7 Hz) was observed at δ 3.17 which can be ascribe to H-6a. The assignment of the aliphatic protons and methylene were also confirmed by analysis of the COSY data. In the COSY spectra (Figure 4.102), H-13 is

coupled to H-14; and H-13 is coupled to H-12β. These match with the suggested structure of **63** having aliphatic protons. Whilst, the respective positions of the protons at C-4 and C-5; and C-7 and C-6a were also confirmed by ^{1}H-^{1}H correlations in the COSY spectrum. Analysis showed that H6a correlated to H7α; and H4β correlated to H5α.

NOE difference spectrum was used to confirmed the position of the methoxyl groups on ring A and ring D. Irradiation on C-10 methoxyl (δ 3.85) showed the enhancement of C-11 (δ 7.80) aromatic proton (2.81%) whereas irradiation of C11 exhibited enhancement of C-10 methoxyl (11.72%) (Figure 4.97), hence this methoxyl group must be attached to C-10. Irradiation of the H-13 signal at δ 3.88, resulted in NOE enhancement of the methyl signal at δ 1.12 (H-14) and the proton signals at δ 3.11 (H-5β) and δ 2.18 (H-12α) (Figure 4.98). When a proton at H-6a was irradiated, the signal of proton H-7β at δ 2.94 (3.71%) was enhanced (Figure 4.99). Irradiation of the proton H-12β at δ 2.70 enhanced the proton signal at H-7α and H-5α (Figure 4.100). The result of NOE-diff experiments supported the finding of the structure of the alkaloid **63**.

The ^{13}C NMR spectrum (Figure 4.101) showed the presence of twenty one carbons in the molecule. These twenty one carbons belonged to one methyl carbon, four methylene carbons, two methines, three aromatic carbons, nine quaternary carbons and two methoxyl carbons as indicated by DEPT 90, DEPT 135 and ^{13}C NMR spectrum (Figure 4.101) of alkaloid **63**.

The absolute configuration at C-6a of alkaloid **63** was deduced as *S* based on the fact that the known alkaloid isolated from the bark and leaf of *Phoebe scortechinii*, norboldine possess an *S* configuration (K. Awang *et al*., 2007; Nakasato T, & Asada, S.,

1966; Guinaudeau, H. *et al.*, 1987). The aporphines of the S configuration will not be oxygenated at C-7 (Guinaudeau, H. *et al*., 1982). Interestingly, from the COSY spectra H6a revealed correlation to H7eq or H7α, suggesting the possibility that H6a is in an equatorial configuration and pointing to a *cis* relationship between H6a and H7eq at δ 2.42 (*J*=3.7 Hz). Finally, the absolute configuration 6a-*S* was assigned to alkaloid **63** on the basis of the positive value of its optical rotation (Bentley & Cardwell, 1955). The stereocenter at C-13 with the *S* configuration (anti clockwise) give a methine proton (H-13) correlate to H-12β or H12ax at δ 2.70 (COSY spectra - Figure 4.102) confirmed the presence of - $CH_2CHOHCH_3$ group attached to the nitrogen in the proposed structure of alkaloid **63.**

Direct correlations between carbon-hydrogen were found from the HMQC spectrum (Figure 4.103) and the results were supported by other data. The structure of alkaloid **63** was finally confirmed by ^{1}H - ^{13}C long range correlations as indicated in the HMBC spectrum (Figure 4.104).

Table 4.15 summarizes the ^{1}H and ^{13}C-NMR chemical shift values and coupling patterns of the proton obtained from various NMR experiments. The compound **63** was identified as (+)-tavoyanine A or *N*-(2-hydroxypropyl)-Norboldine A based on the above spectral data and comparison with literature reports of compound (Shamma & Slusarchyk, 1964; Borthakur & Rastogi, 1979; Guinaudeau *et al*., 1975; Guinaudeau *et al.*, 1983; Guinaudeau *et al*., 1994). To the knowledge of the author, (+)-tavoyanine A (**63**) is the first report as new naturally occurring compound.

Table 4.15: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT data and 2D (HMBC and HMQC) NMR data of (+)-tavoyanine A (**63**).

63 in $CDCl_3$					
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	DEPT	HMBC (^{2}J,^{3}J)	HMQC (^{1}J)
1	-	142.2	C_q	-	-
1a	-	126.6	C_q	-	-
1b	-	127.3	C_q	-	-
2	-	148.2	C_q	-	-
3	6.58 (*s*, 1H)	113.3	CH	C_1,C_{1b}, C_2, C_4	H_3
3a	-	130.4	C_q	-	-
4β	2.60 (*d*, *J*=4.3)	29.4	CH_2	C_{1b}, C_{3a}, $C_{5.}$	H_4
4α	2.56 (*d*, *J*=4.3)			C_{1b}, C_3, C_{3a}.	
5β	3.11 (*dd*, *J*=4.1, 11.3)	49.4	CH_2	C_{3a},C_4,C_{6a}	H_5
5α	2.38 (*dd*, *J*= 4.1, 11.3)			C_{3a}, C_{6a}	
6a	3.17 (*dd*, *J*= 3.2, 13.7)	60.4	CH	-	H_{6a}
7β	2.94 (*dd*, *J*= 3.7, 12.0)	34.9	CH_2	C_{1a},C_{6a},C_{7a},C_8,C_{11a}	H_7
7α	2.42 (*dd*, *J*= 3.7, 12.0)			C_{1b},C_{6a}, C_8,C_{11a}	
7a	-	130.2	C_q	-	-
8	6.76 (*s*, 1H)	114.3	CH	C_7,C_{10}, C_{11a}	H_8
9	-	145.3	C_q	-	-
10	-	145.8	C_q	-	-
11	7.80 (*s*, 1H)	110.3	CH	C_{1a}, C_{7a}, $C_{9,}$ C_{11a}	H_{11}
11a	-	123.7	C_q	-	-
12β	2.70 (*dd*, *J*= 2.7, 12.6)	61.5	CH_2	C_5, C_{6a}, $C_{13,}$ $C_{4.}$	H_{12}
12α	2.18 (*dd*, *J*= 2.7, 12.6)			$C_{5.}$	
13	3.88 *m*	62.9	CH	-	H_{13}
14	1.12 (*d*, *J*= 6.4)	19.8	CH_3	C_{12}, C_{13}	$3H_{14}$
1-OCH_3	3.50 (*s*, 3H)	60.4	CH_3	C_1	$3H_{1\text{-}OMe}$
10-OCH_3	3.85 (*s*, 3H)	56.4	CH_3	C_{10}	$3H_{10\text{-}OMe}$

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

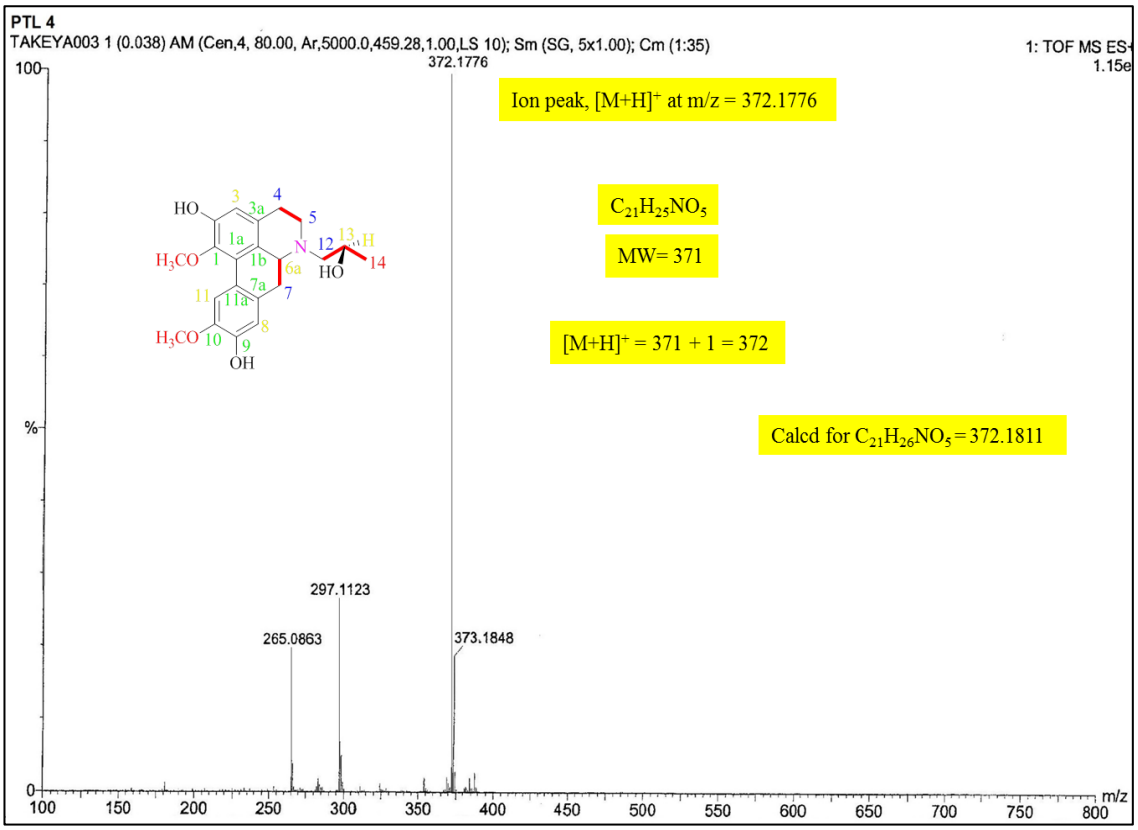


Figure 4.94: LCMS- ESI spectrum of tavoyanine A (**63**).

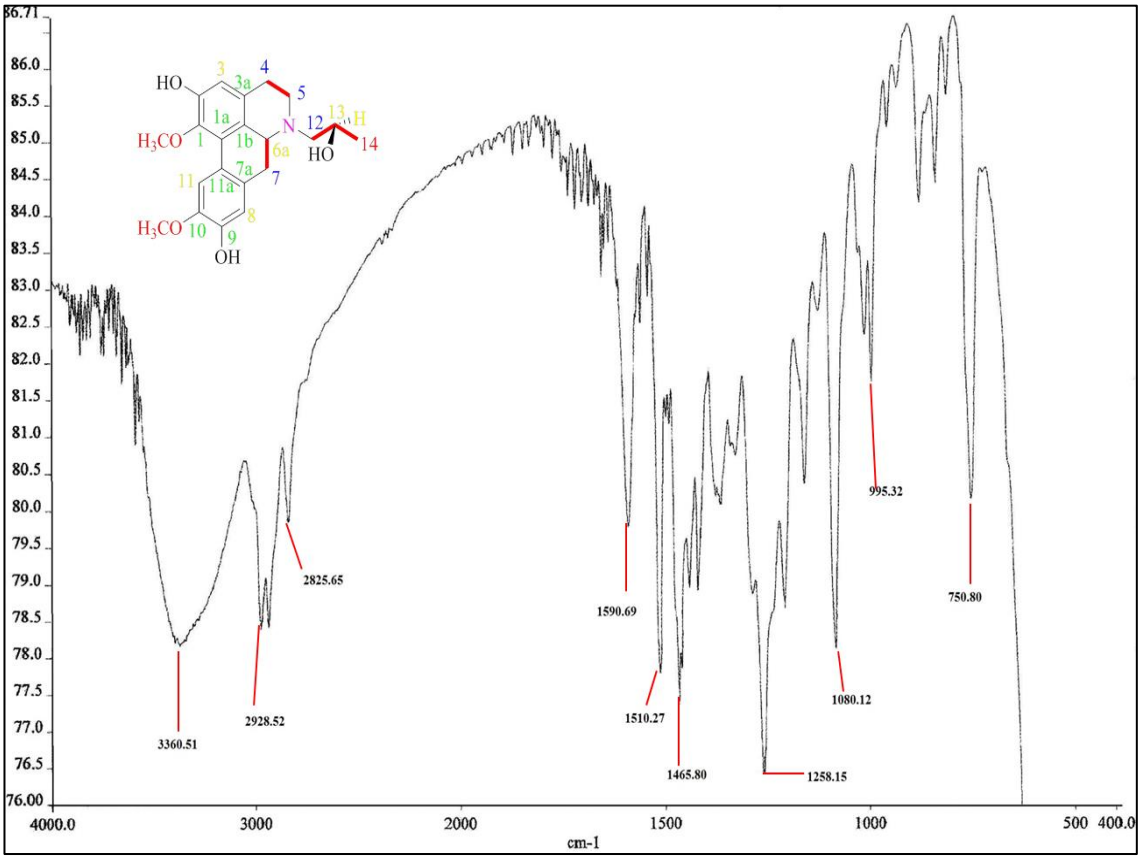


Figure 4.95: IR spectrum of tavoyanine A (**63**).

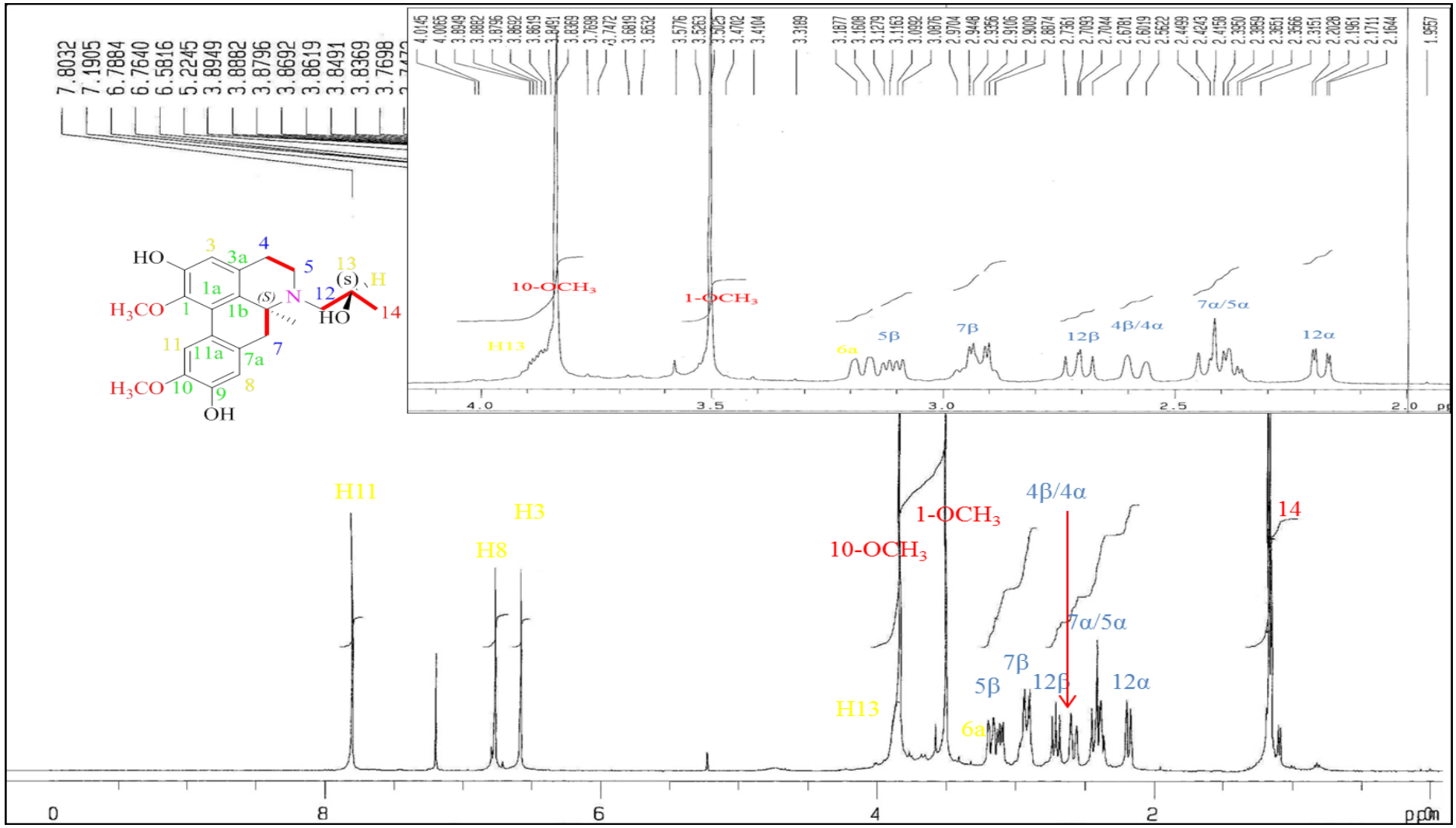


Figure 4.96: ^{1}H- NMR spectrum of tavoyanine A (**63**).

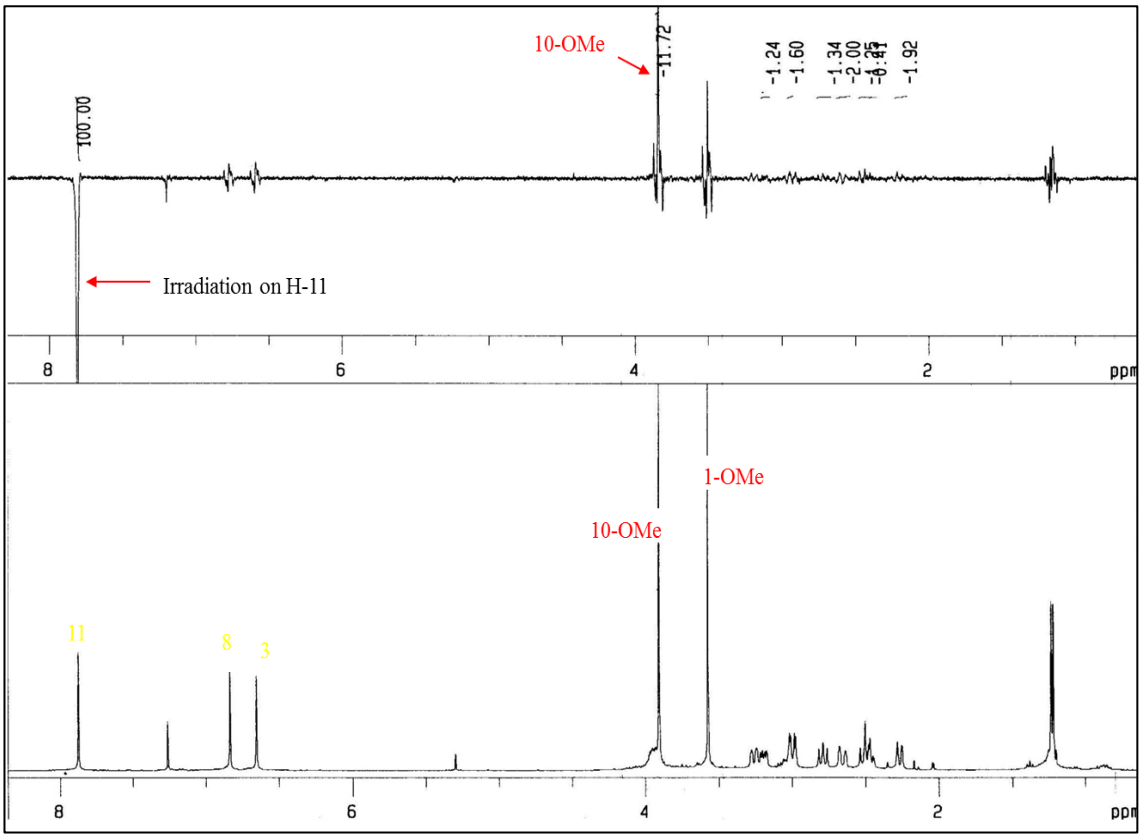


Figure 4.97: ^{1}H NOE – Differential-1 spectrum of tavoyanine A (**63**).

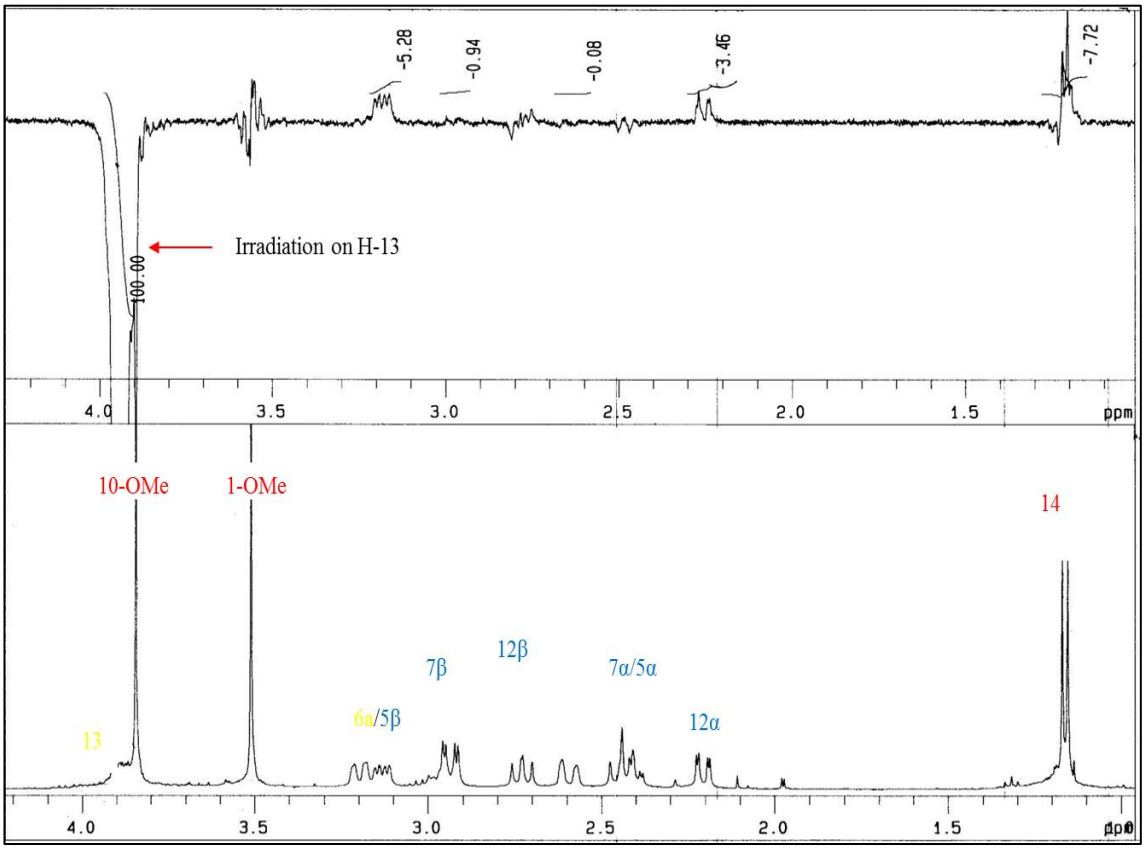


Figure 4.98: ^{1}H NOE – Differential-2 spectrum of tavoyanine A (**63**).

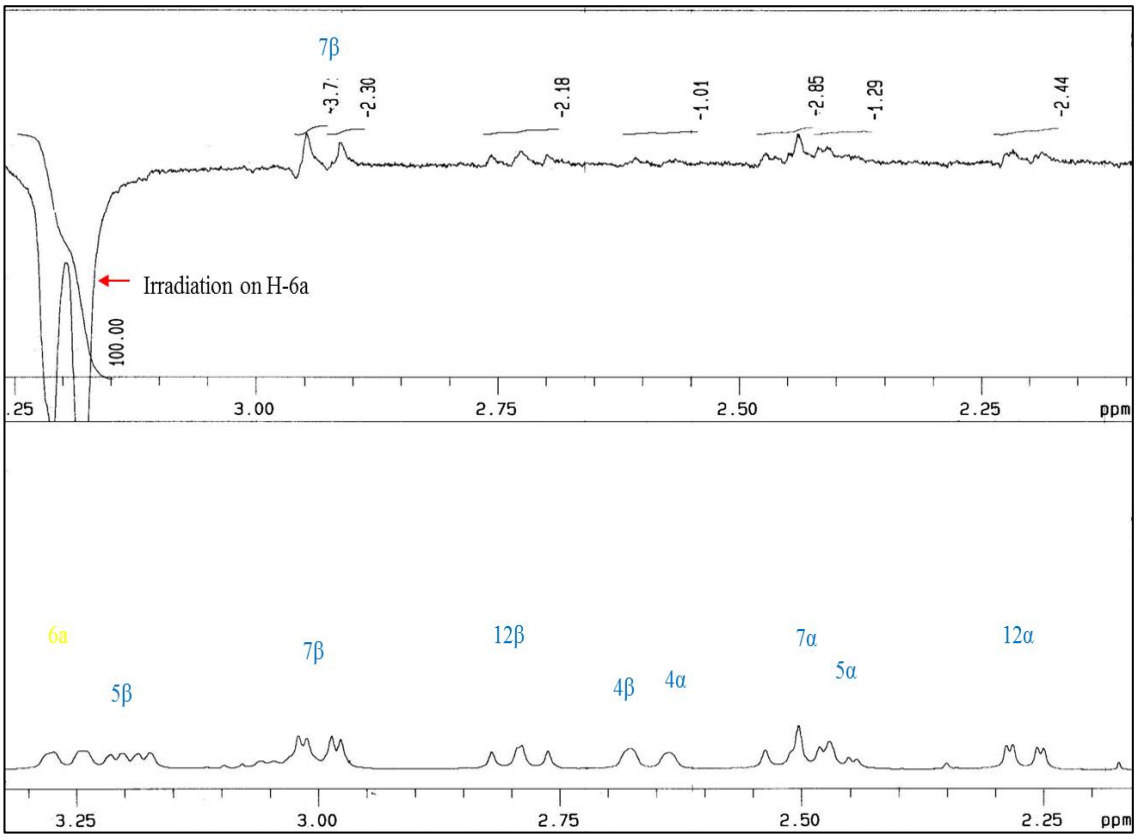


Figure 4.99: ^{1}H NOE – Differential-3 spectrum of tavoyanine A (**63**).

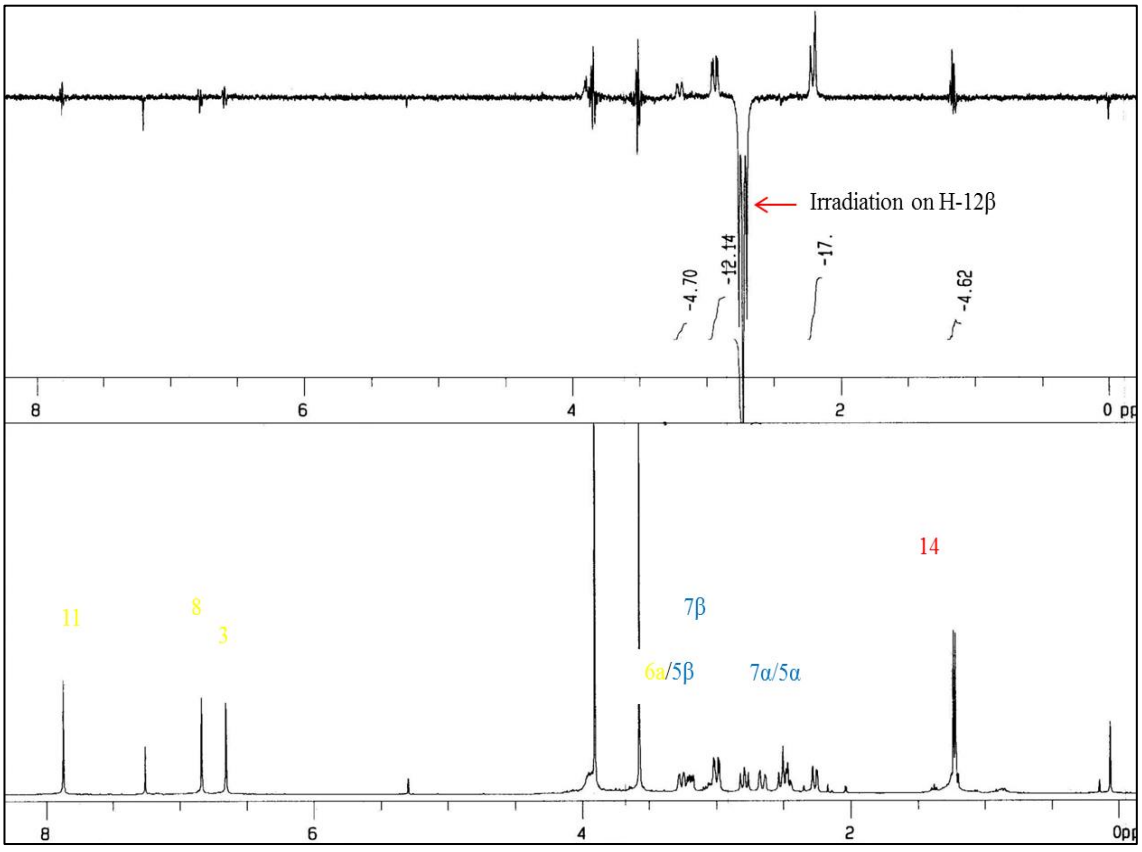


Figure 4.100: ^{1}H NOE – Differential-4 spectrum of tavoyanine A (**63**).

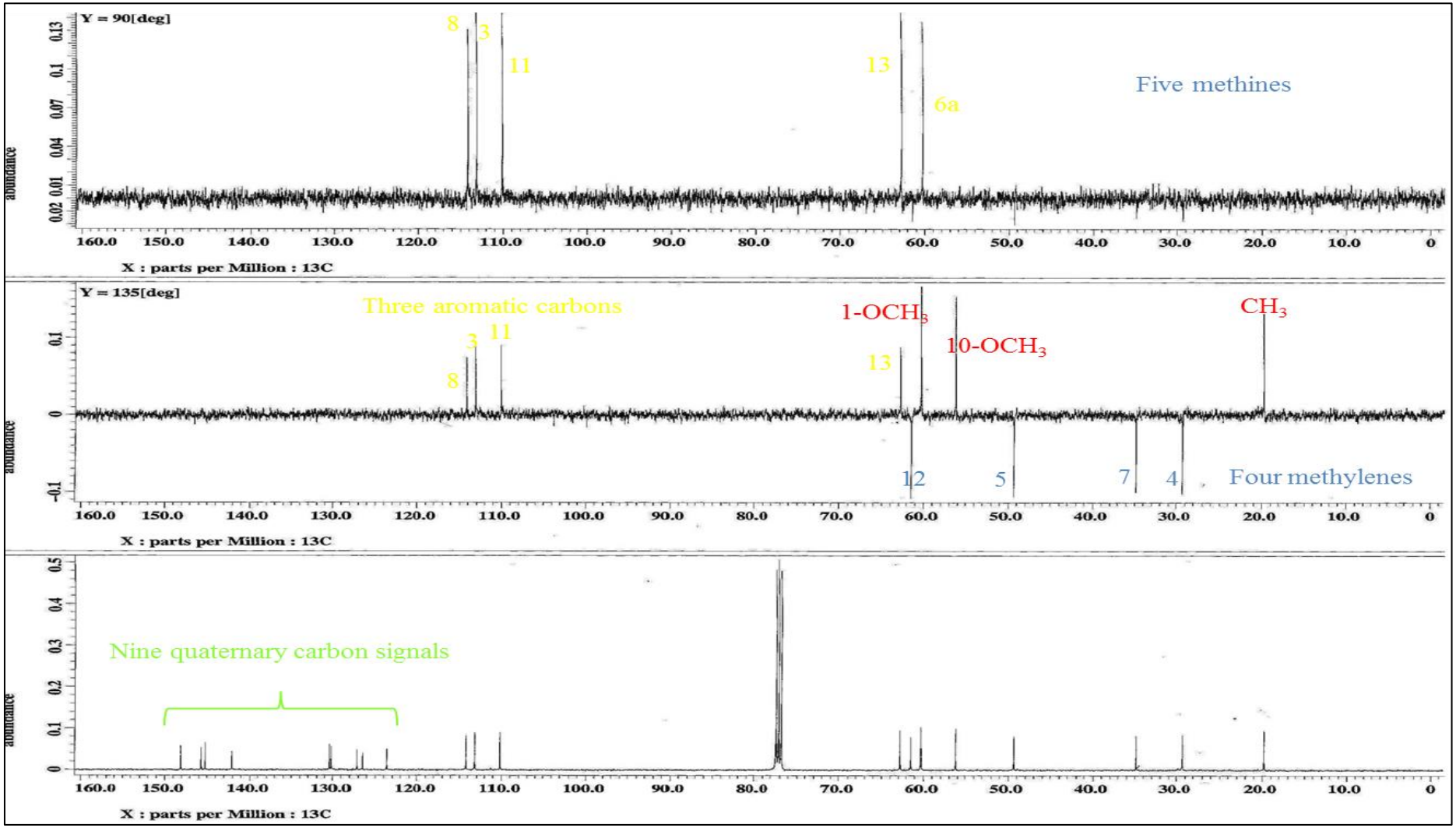


Figure 4.101: DEPT 90, DEPT 135 and ^{13}C NMR spectrum of tavoyanine A (**63**).

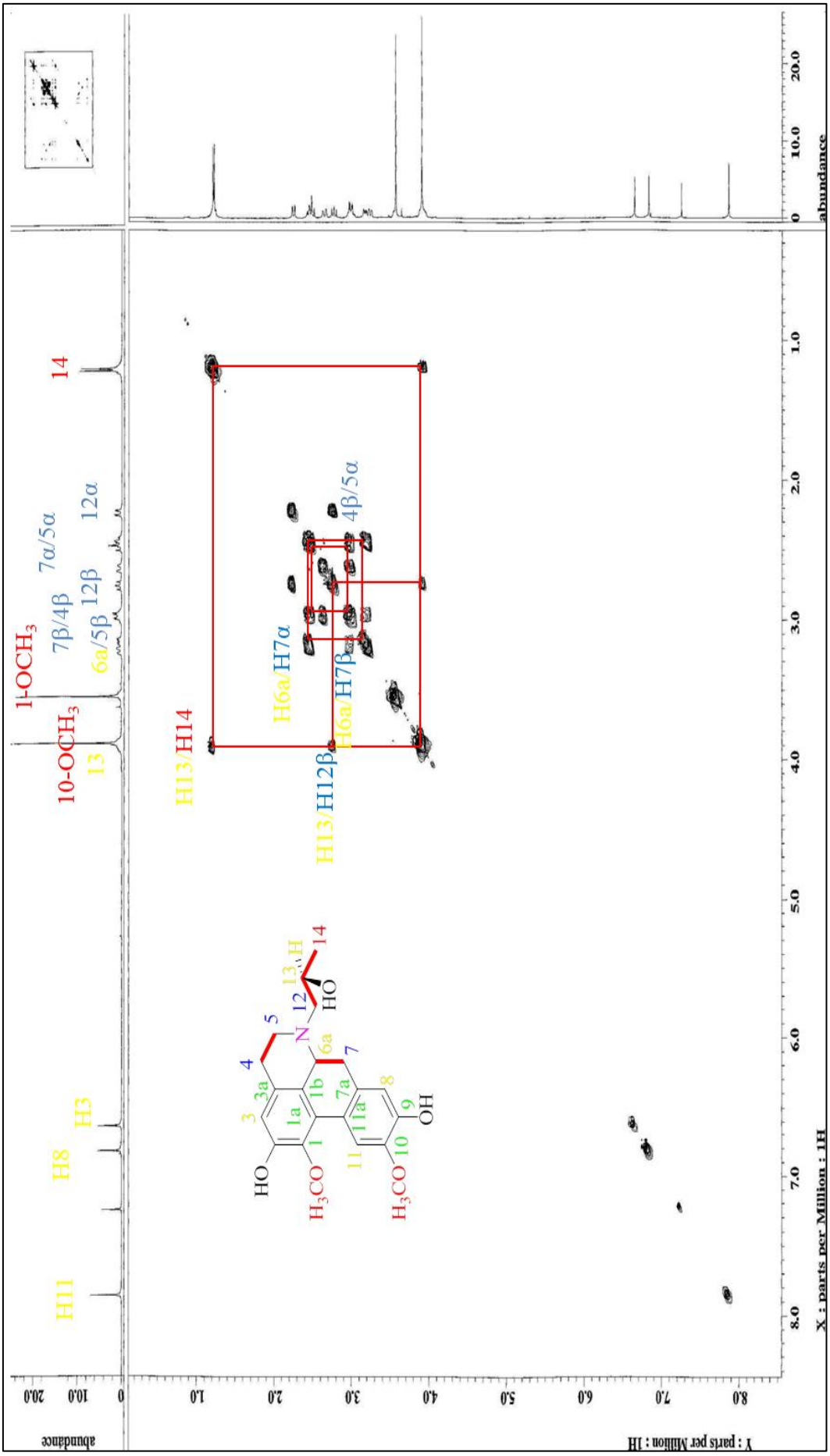


Figure 4.102: COSY spectrum of tavoyanine A (**63**).

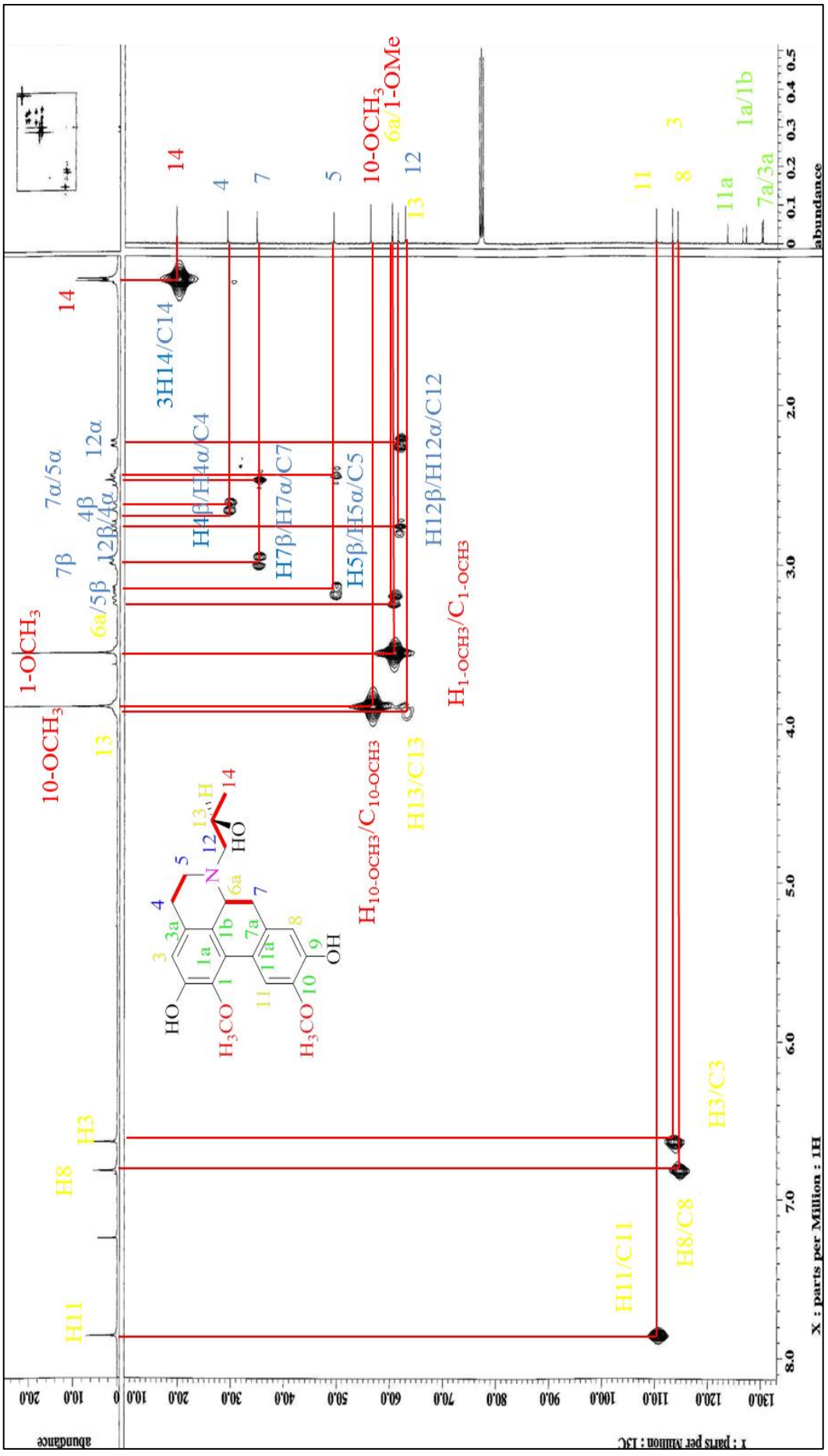


Figure 4.103: HMQC spectrum of tavoyanine A (**63**).

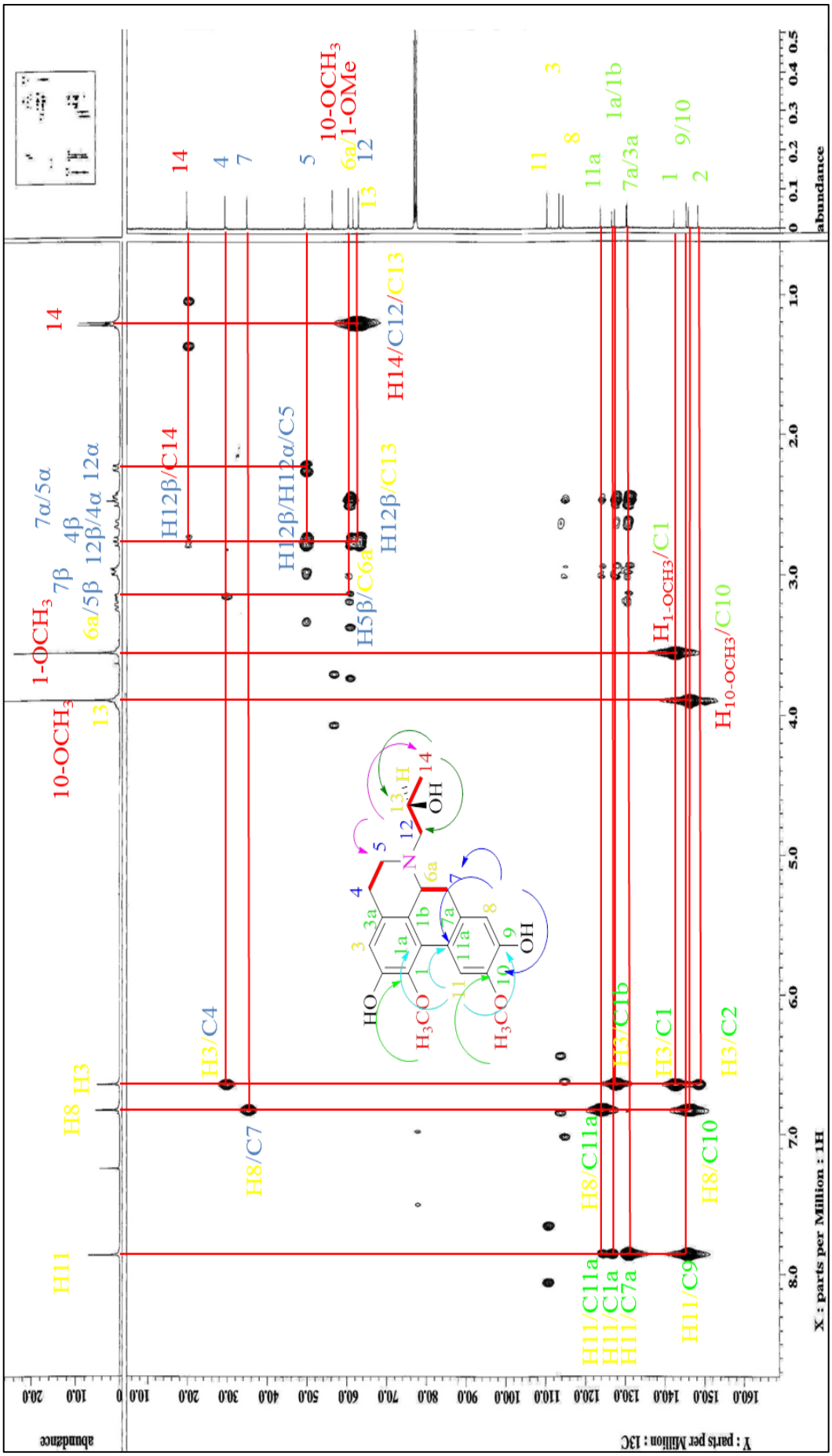
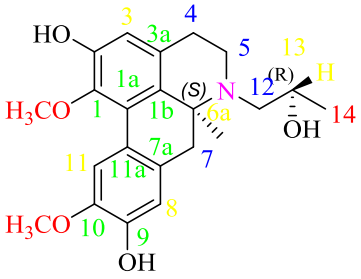


Figure 4.104: HMBC spectrum of tavoyanine A (**63**).

4.4.5 Tavoyanine B (64)



64

Alkaloid **64** was obtained as a brownish amorphous solid. $[\alpha]_D^{25}$ = - 0.03°(c=0.5, CH_3OH). The molecular formula was deduced as $C_{21}H_{25}NO_5$ based on the LCMS-ESI mass spectrometry (Figure 4.105), which gave a molecular ion peak at m/z 372.1804 $[M+H]^+$, (calcd. for $C_{21}H_{26}NO_5$, 372.1811). The UV absorptions at 293 nm and 304 nm, suggested the presence of 1,2,9,10-tetrasubstituted aporphine skeleton (Goodwin *et al*., 1958) in the structure. Its IR spectrum (Figure 4.106), showed the presence of a broad band at 3300 cm^{-1} due to the stretching of OH group. In addition, its IR spectrum also showed strong absorptions at 2935 cm^{-1} and 2838 cm^{-1} due to the stretching of C-H aromatic, respectively. While, the absorptions at 1453 and 1568 cm^{-1} indicated aromatic C=C stretching.

The ^1H NMR spectrum (Figure 4.107) showed two sharp singlets corresponding to six methoxyl protons observed at δ_H 3.59 and δ_H 3.92. Three aromatic proton singlets were observed at δ 6.65, 6.82 and 7.88. The latter was assigned to H-11 and the other aromatic protons attributed to H-3 and H-8, respectively. Thus, it can conclude that the two methoxyl groups were located at C-1 and C-10 in ring A and D. A pair of doublet (*J* = 3.9 and 13.9 Hz) corresponding to one proton were observed at δ 3.40 (H-6a). In addition, a multiplet signal were observed at δ 3.85 (*m*) due to proton at C-13. Furthermore, the spectrum also revealed the resonances of a pair of doublet of doublet

at δ 2.88 (*dd, J*=3.9, 13.6 Hz) and δ 2.36 (*dd, J*=9.3, 13.6 Hz) due to H12β and H12α. A three protons doublet at δ 1.20 (*J*=6.1 Hz) indicated the existence of a methyl group next to a methine proton which is part of – $CH_2CHOHCH_3$ moiety.

NOE difference spectrum was used to confirm the position of the methoxyl groups on ring A and D. Irradiation of the methoxyl group at δ3.92 (Figure 4.108) enhanced the proton signal at δ 2.62 (H-7α) methylene proton (4.53%) and δ 7.88 (H-11) aromatic proton (3.31%), hence this methoxyl group must be attached to C-10. On the other hand, irradiation at δ 6.82 (H-8) (Figure 4.109) resulted in NOE enhancement of the methylene proton signal at δ 2.88 (H-12β) 7.25% and δ2.79 (H-7β) 4.78%. No methoxyl singlet was enhanced, hence suggesting that a hydroxyl attached to C-9. The irradiation of the proton H-14 at δ 1.20 (Figure 4.110) enhanced the proton signal of H-13 (δ 3.85) 9.46 %, H-6a (δ 3.40) 7.25 %, H-7α (δ 2.62) 6.19 % and H-7β (δ 2.79) 3.83% confirming the isopropyl moiety attached to N of aporphine skeleton. While, irradiation of the proton H-3 at δ 6.65 (Figure 4.111) enhanced the methoxyl signal C-1 at δ 3.59 (16.91%) and the proton signal H-4α at δ 2.66 (6.79 %), suggesting that hydroxyl attached to C-2. The relative configuration at C-13 spiro carbon could be considered a diastereomeric compound of **63**. The position C-13 of **64** appeared at δ 66.0 whereas C-13 of **63** appeared at δ 62.9.

The ^{13}C NMR spectrum (Figure 4.112) revealed the presence of 21 carbon peaks, including a methyl, four methylenes, two methoxyl groups, two methines, three unsubstituted aromatic carbons and nine quaternary carbons as indicated by DEPT and ^{13}C NMR spectrum (Figure 4.112). It could be deduced that **64** had methoxyl group at C-1 and C-10; and methyl group at position C-14. The position of these groups was established by HMBC experiment (Figure 4.114). The methoxyl signals at δ 3.59

correlated with the C-1 signal (δ_c 142.1) and δ 3.92 correlated with the C-10 signal (δ_c 145.7). Furthermore, the methyl signal at δ 1.20 was correlated with the methylene carbon at δ 63.3 (C-12) and methine carbon at δ 66.0 (C-13).

The aporphines of the *S* configuration will not be oxygenated at C-7 (Guinaudeau, H. *et al*., 1982). The absolute configuration at C-6a of alkaloid **64** was deduced as *S* based on the fact that the known alkaloid isolated from the bark and leaf of *Phoebe scortechinii*, norboldine possess an *S* configuration (K. Awang *et al*., 2007; Nakasato T, & Asada, S., 1966; Guinaudeau, H. *et al.,* 1987). On the basis of the negative value of its optical rotation (Bentley & Cardwell, 1955), the stereocenter at C-13 indicative isopropyl possess the *R* configuration.

The placement of all protons and quaternary carbons were confirmed by the ^{1}H-^{13}C direct correlations in HMQC spectrum (Figure 4.113) and ^{1}H-^{13}C long range correlations observed in the HMBC spectrum (Figure 4.114). The complete assignment of all protons and carbons were listed in Table 4.16. The structure was finally confirmed by comparison with the literature reports of known compound, norboldine (Shamma & Slusarchyk, 1964; Borthakur & Rastogi, 1979; Guinaudeau *et al*., 1975; Guinaudeau *et al.*, 1983; Guinaudeau *et al*., 1994). These results indicate that alkaloid **64** is a new naturally occuring aporphine alkaloid and was isolated for the first time from *Phoebe* genus. The structure of this compound **64** was assigned the trivial name (-)-tavoyanine B (**64)** or *N*-(2-hydroxypropyl)-Norboldine B.

Table 4.16: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), DEPT and 2D (HMBC and HMQC) NMR data of tavoyanine B (**64)**.

64 in $CDCl_3$					
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	DEPT	HMBC (^2J,^3J)	HMQC (^1J)
1	-	142.1	C_q	-	-
1a	-	126.2	C_q	-	-
1b	-	127.2	C_q	-	-
2	-	148.1	C_q	-	-
3	6.65 (*s*, 1H)	113.3	CH	C_1,C_{1b}, C_2, C_4	H_3
3a	-	130.2	C_q	-	-
4β	3.00 *m*	29.0	CH_2	C_{3a}, $C_{5.}$	H_4
4α	2.66 *m*			C_{1b}, C_{3a}, $C_{5.}$	
5β	3.06 *m*	52.8	CH_2	C_{3a}, C_4, C_{6a}	H_5
5α	2.83 *m*			C_{3a}, C_4, C_{6a}	
6a	3.40 (*dd*, *J*=3.9, 13.9)	61.4	CH	-	H_{6a}
7	2.79 *m*	35.3	CH_2	C_{1b},C_{6a},C_{7a},C_8,C_{11a}	H_7
	2.62 *m*			C_{1b},C_{6a},C_{7a}	
7a	-	130.3	C_q	-	-
8	6.82 (*s*, 1H)	114.3	CH	C_7,C_{10}, C_{11a}	H_8
9	-	145.2	C_q	-	-
10	-	145.7	C_q	-	-
11	7.88 (*s*, 1H)	110.1	CH	C_{1a}, C_{7a}, $C_{9,}$ C_{11a}	H_{11}
11a	-	123.6	C_q	-	-
12β	2.88 (*dd*, *J*=3.9, 13.6)	63.3	CH_2	C_4, C_{6a}.	H_{12}
12α	2.36 (*dd*, *J*=9.3, 13.6)			$C_{5,}$ $C_{6a,}$ $C_{13,}$ $C_{14.}$	
13	3.85 *m*	66.0	CH	-	H_{13}
14	1.20 (*d*, *J*=6.1)	20.6	CH_3	C_{12}, C_{13}	$3H_{14}$
1-OCH_3	3.59 (*s*, 3H)	60.4	CH_3	C_1	$3H_{1\text{-OMe}}$
10-OCH_3	3.92 (*s*, 3H)	56.3	CH_3	C_{10}	$3H_{10\text{-OMe}}$

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

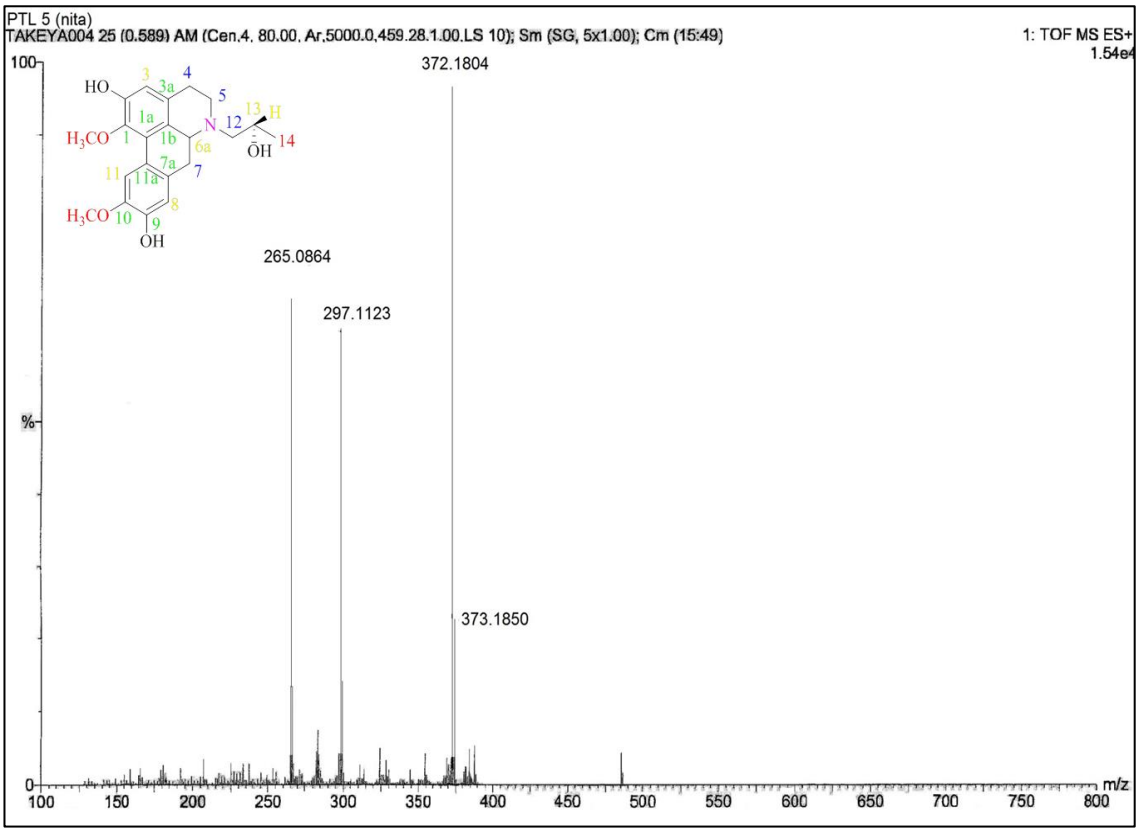


Figure 4.105: LCMS- ESI spectrum of tavoyanine B (**64**).

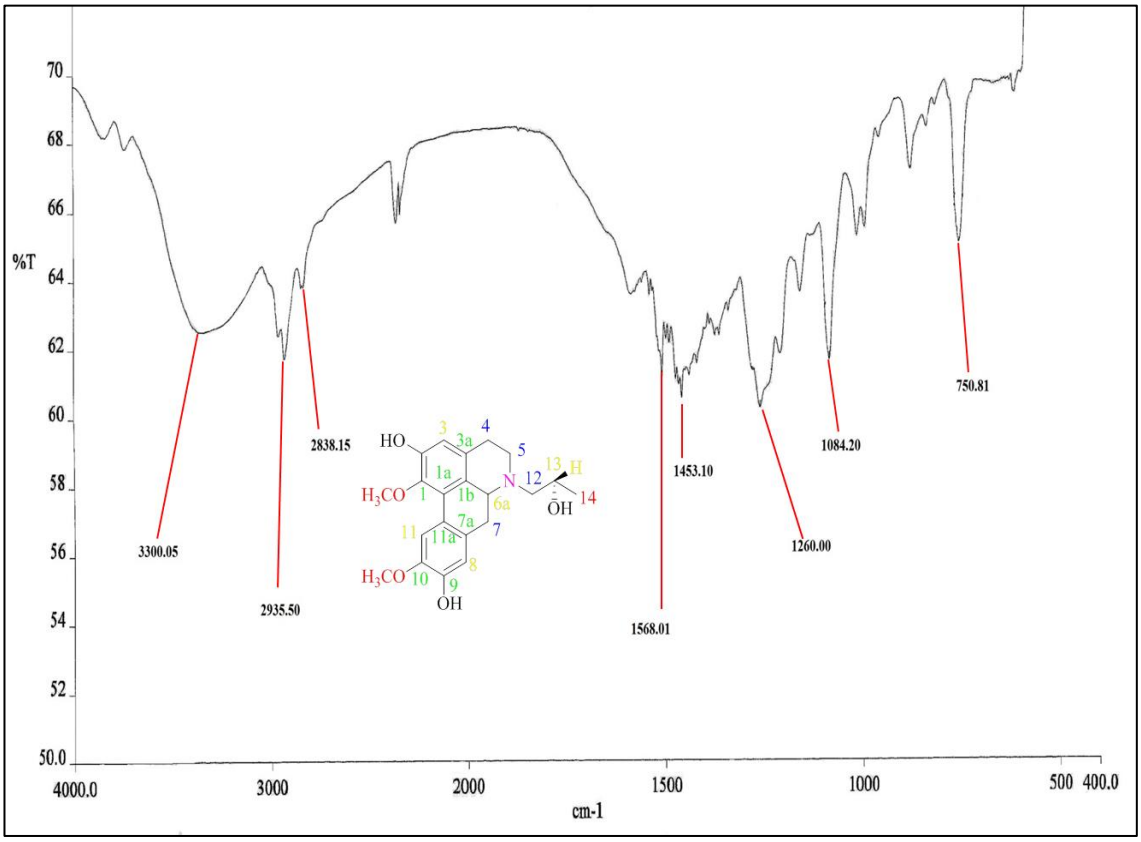


Figure 4.106: IR spectrum of tavoyanine B (**64**).

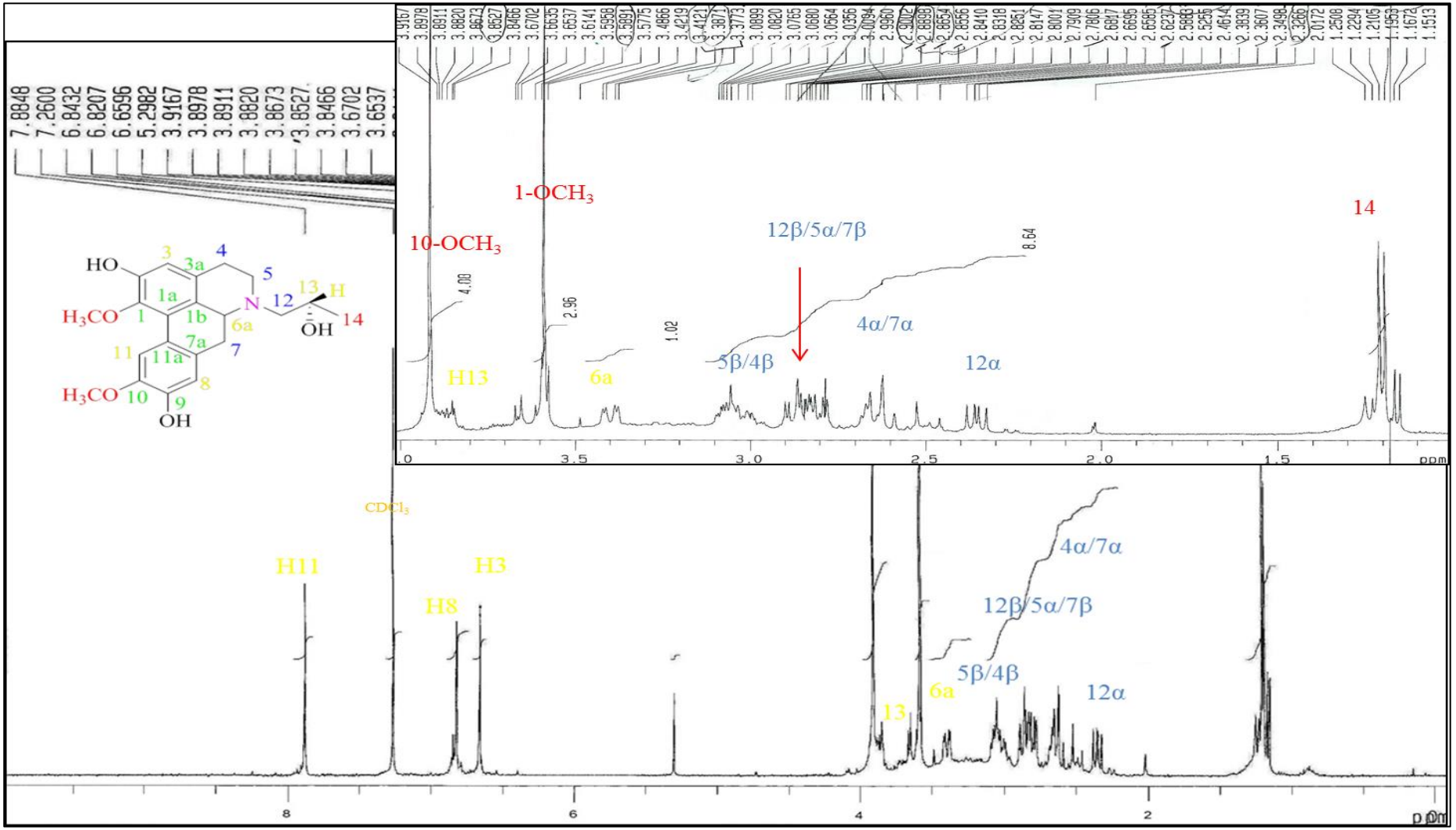


Figure 4.107: ^{1}H- NMR spectrum of tavoyanine B (**64**).

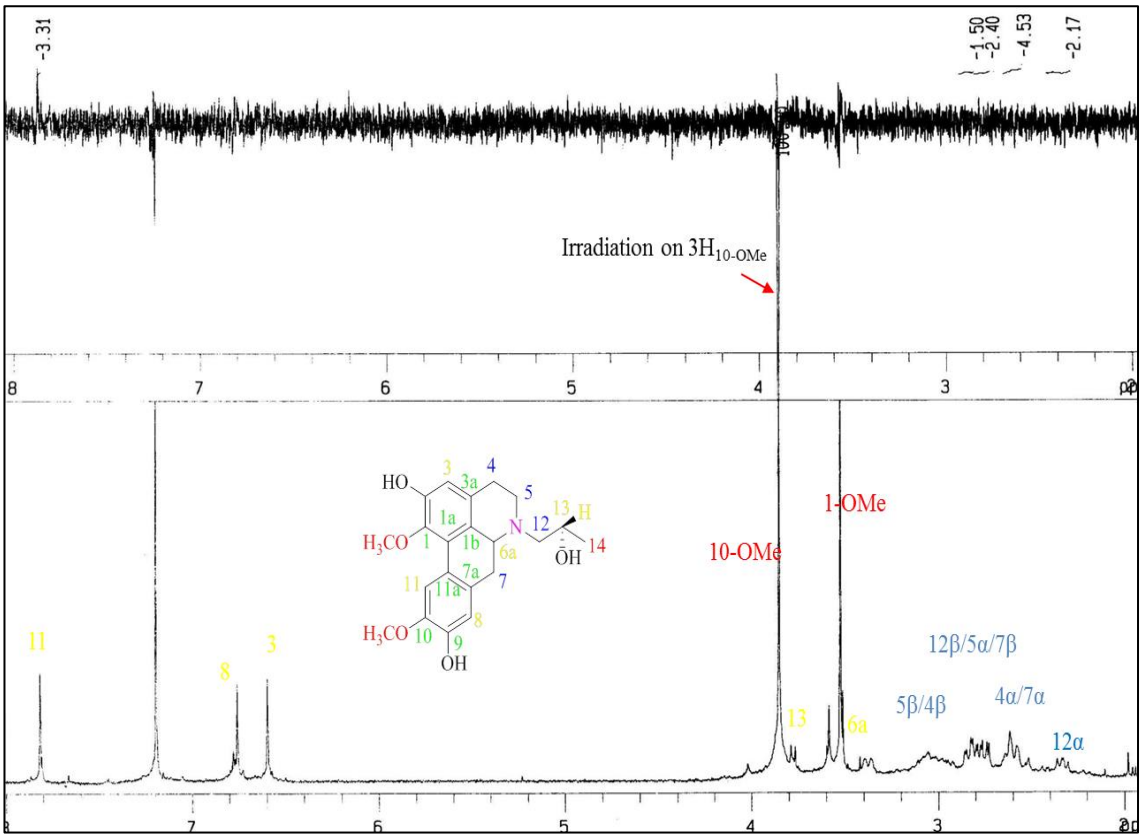


Figure 4.108: ^{1}H NOE – Differential-1 spectrum of tavoyanine B (**64**).

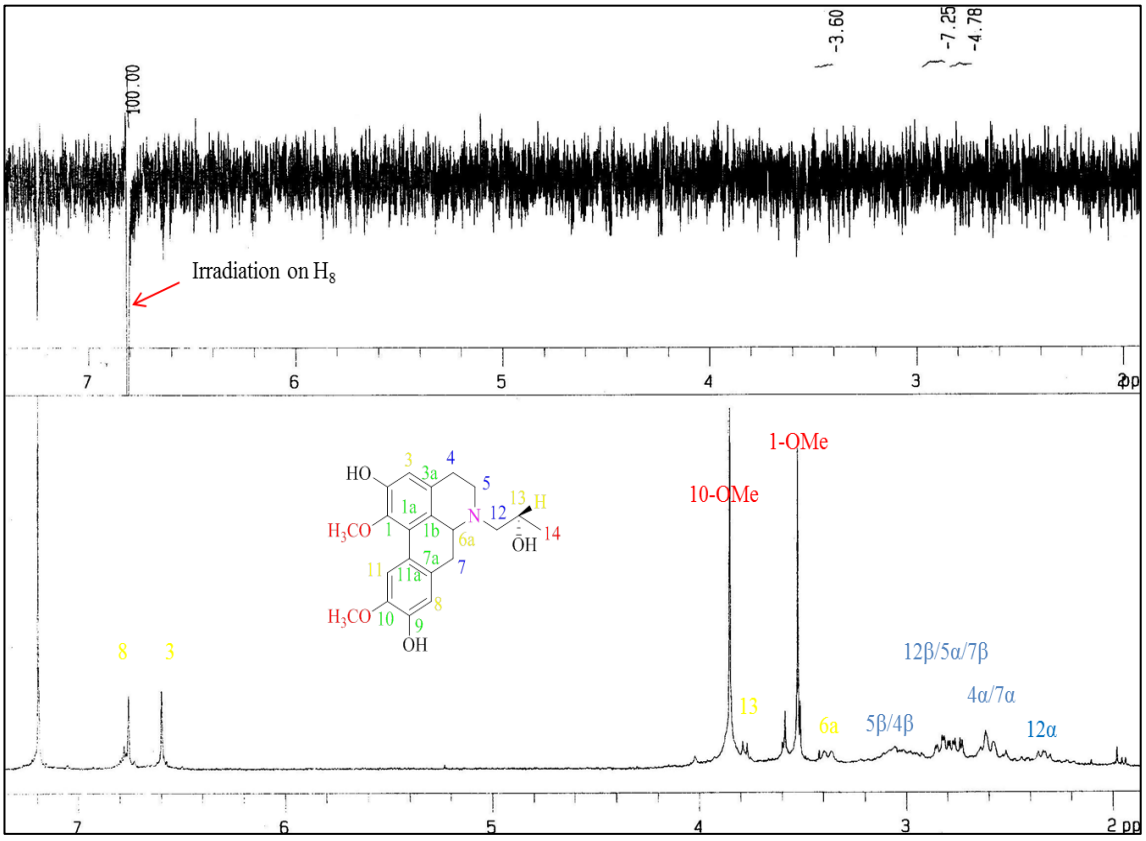


Figure 4.109: ^{1}H NOE – Differential-2 spectrum of tavoyanine B (**64**).

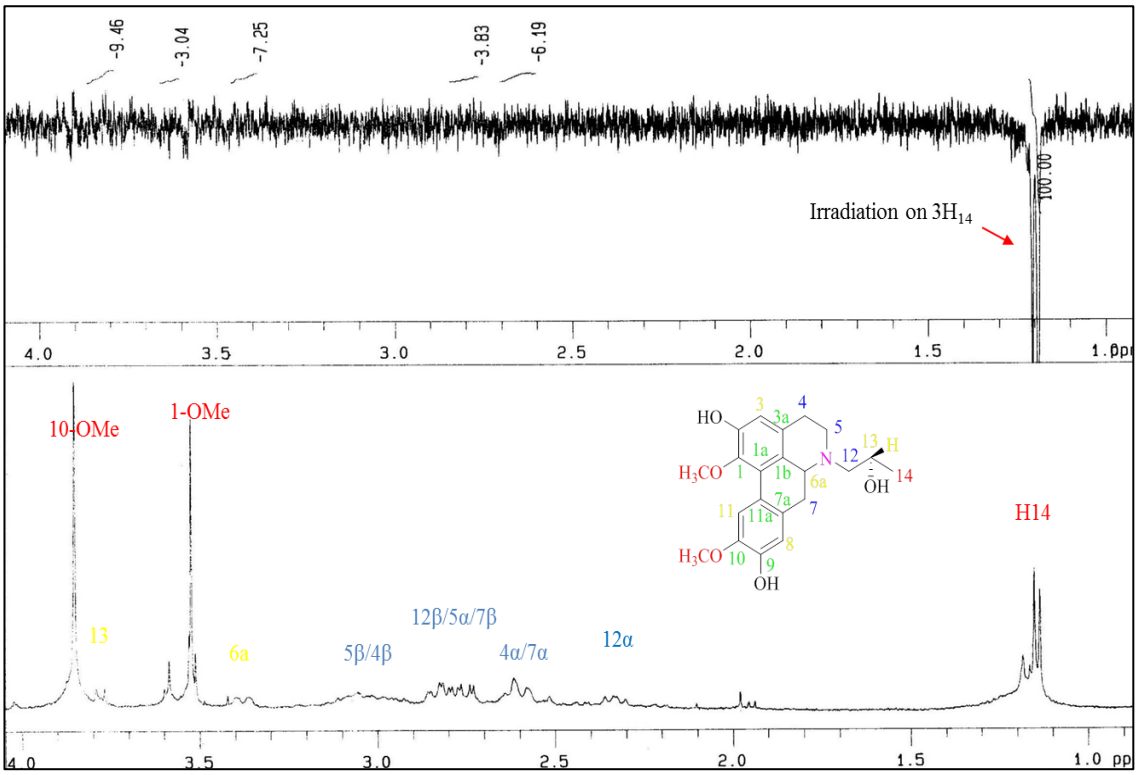


Figure 4.110: ^{1}H NOE – Differential-3 spectrum of tavoyanine B (**64**).

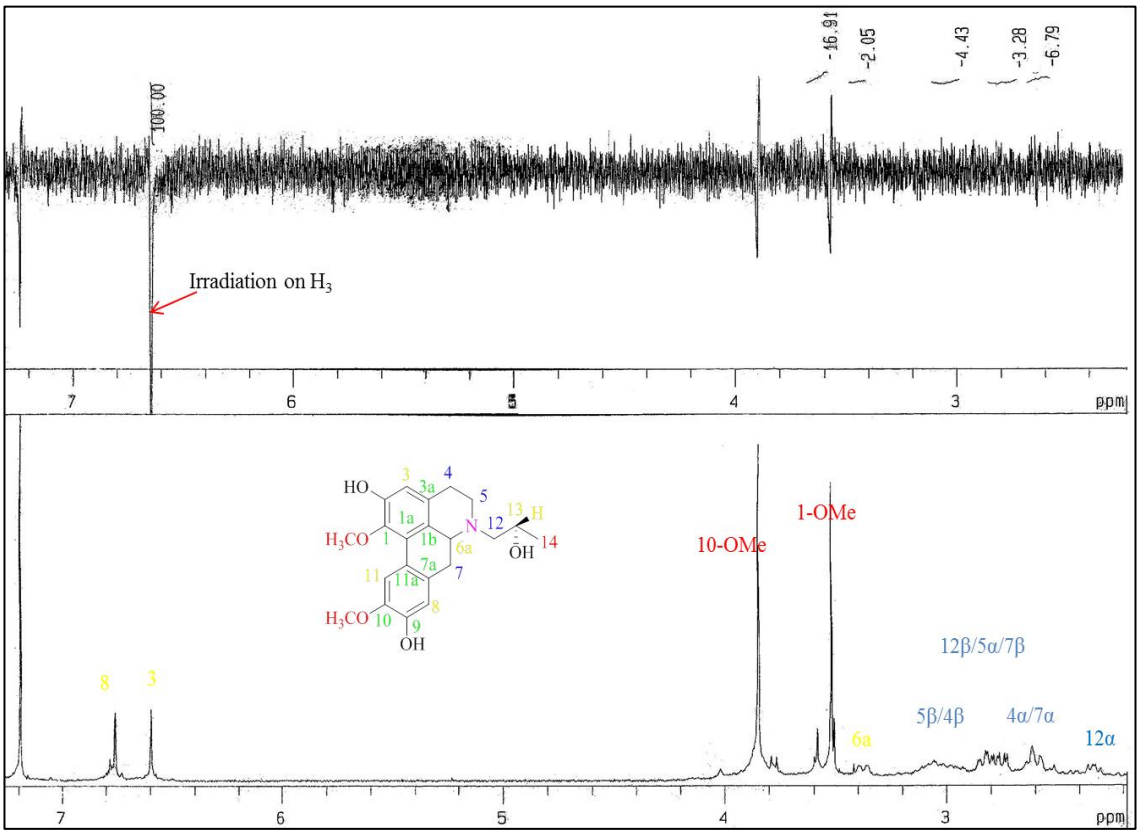


Figure 4.111: ^{1}H NOE – Differential-4 spectrum of tavoyanine B (**64**).

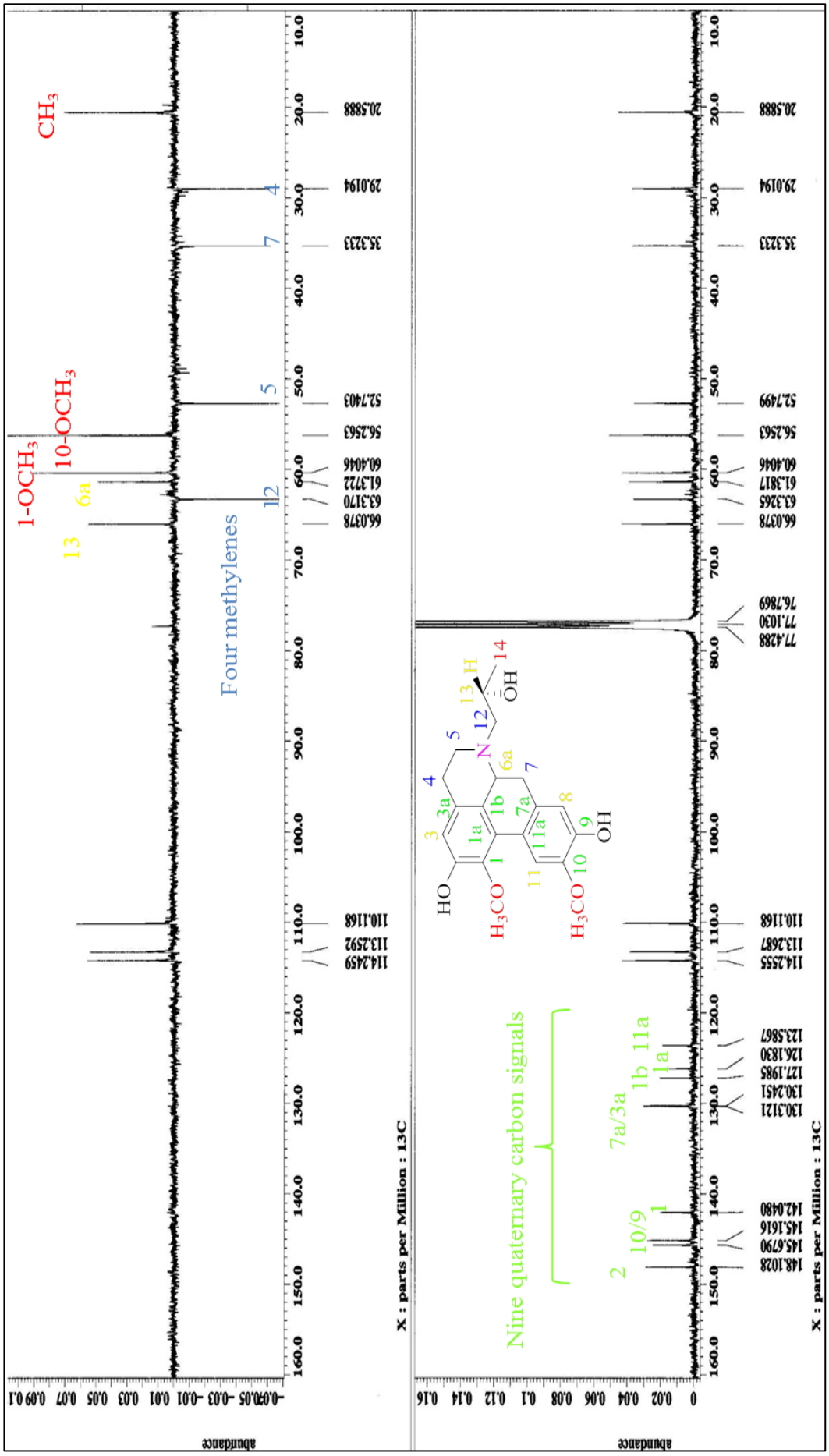


Figure 4.112: DEPT and ^{13}C NMR spectrum of tavoyanine B (**64**).

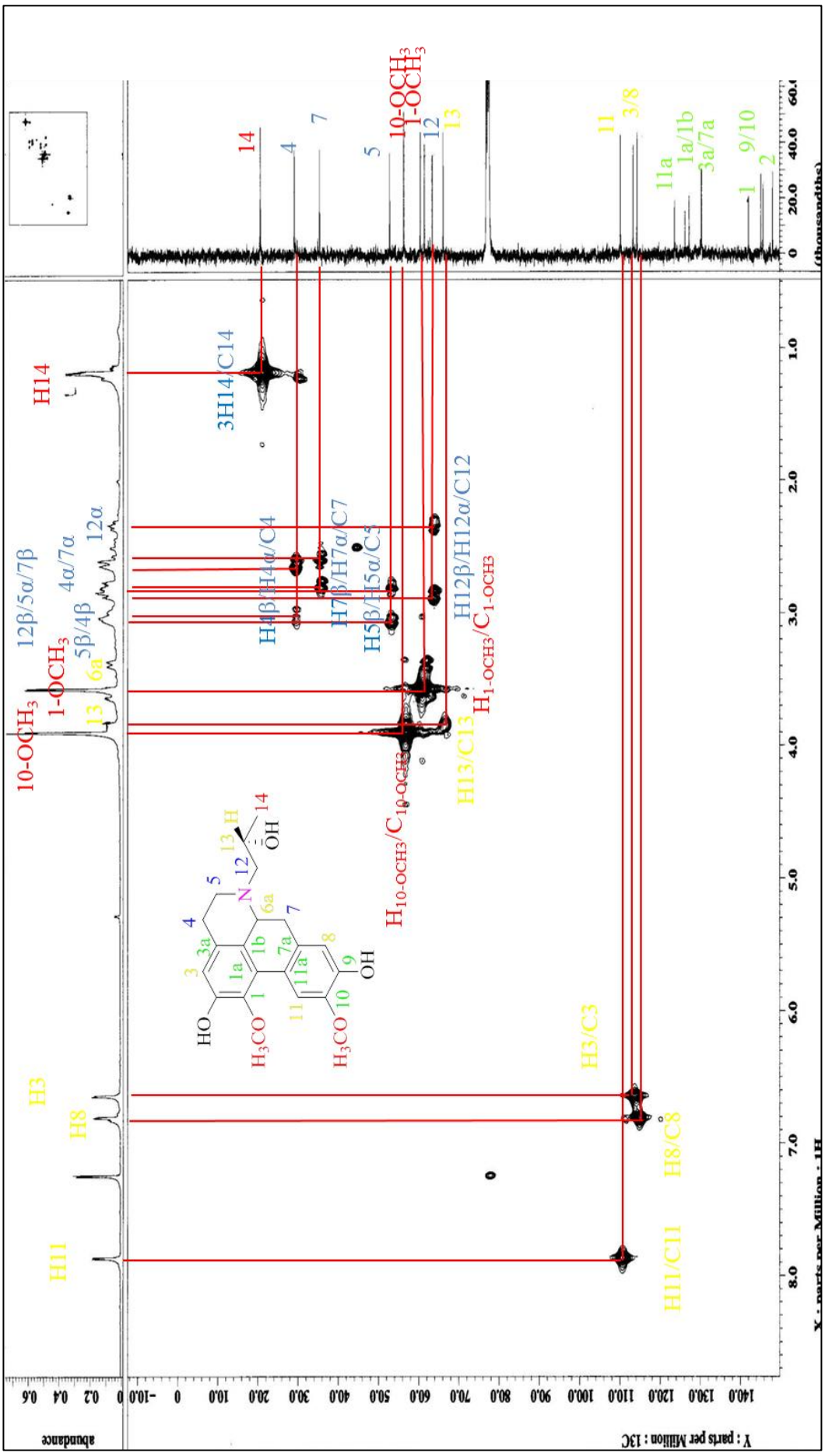


Figure 4.113: HMQC spectrum of tavoyanine B (**64**).

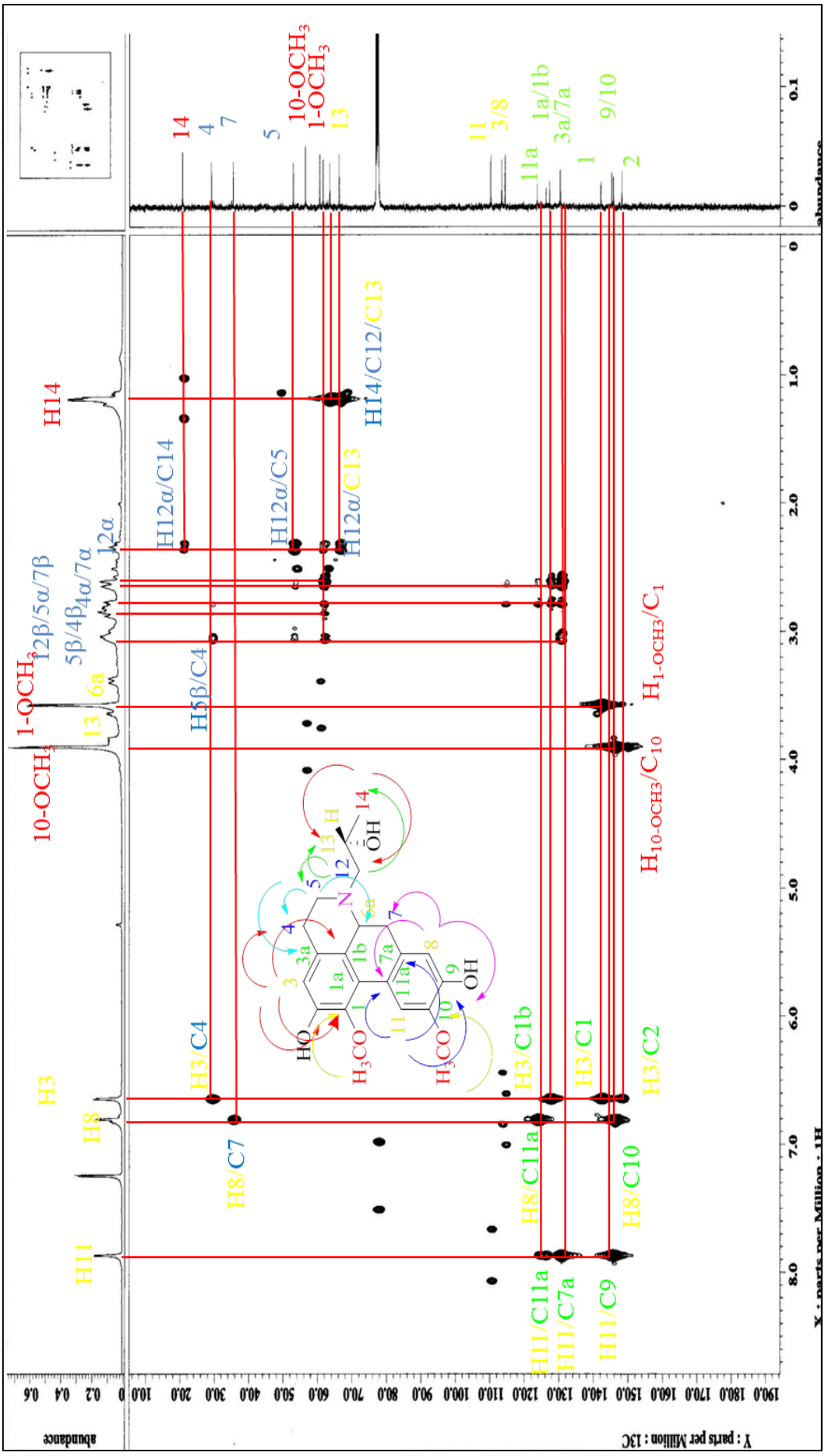
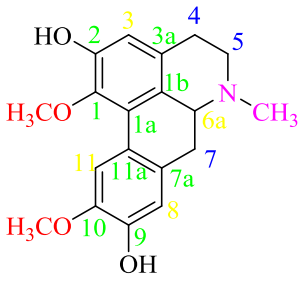


Figure 4.114: HMBC spectrum of tavoyanine B (**64**).

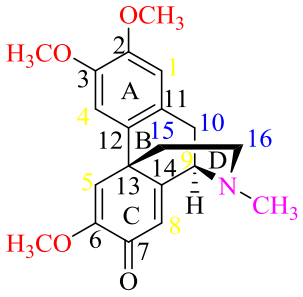
4.4.6 Boldine (5)



5

Alkaloid **5** was isolated as a brownish amorphous solid. The UV, IR, 1D-NMR (^{1}H NMR and ^{13}C NMR) data spectra and NOE different experiment features were similar to those of boldine (**5**) isolated from dichloromethane extract of *Phoebe grandis*. Please refer to **4.3.3** boldine, **5** (page 176-182).

4.4.7 Sebiferine (22)



22

Alkaloid **22** was obtained as a pale yellow amorphous solid, $[\alpha]_D^{25}$ = + 1.38° (c = 0.5 $CHCl_3$), with the melting point of 111-113˚C. The LCMS-ESI spectrum (Figure 4.115) showed a molecular ion peak at m/z 342.1692 $[M+H]^+$ which corresponding to molecular formula of $C_{20}H_{23}NO_4$ (calcd. mass for $C_{20}H_{24}NO_4$, 342.1627). The UV spectrum showed three peaks at 209, 238 and 280 nm. IR spectrum (Figure 4.116**)** showed absorption band at 1660, 1639 and 1615 cm^{-1} gave the characteristic of α-

methoxy cross-conjugated cyclohexadienone. A strong peak at 1660 cm^{-1} together with a quaternary signal at δ180.3 in the ^{13}C- NMR spectrum (Figure 4.118**)** suggested the presence of a quinoid moiety (Bhakuni *et al.,* 1978).

The ^{1}H-NMR spectrum (Figure 4.117**)** showed the presence of four downfield singlets which were related to the aromatic and the cross-conjugated cyclohexadienone protons. There were two *para* oriented aromatic protons at δ 6.77 (*s*, H-4) and δ 6.58 (*s*, H-1) and two cyclohexadienone protons at δ 6.32 (*s*, H-5) and δ 6.22 (*s*, H-8). The H-8 signal was more upfield than H-5 due to the steric effect caused by ring B. H-5 was more deshielded than H-8 due to the delocalisation of α electron from the conjugated double bond. It has been reported that the most shielded aromatic proton in sebiferine is the vinylic proton at position H-8 (Marsaioli *et al*., 1980; Tomita, 1952; Chouna *et al*., 2009). Three singlets of methoxyl groups also appeared at δ 3.82, 3.78 and 3.65 which most probably attached to C-2, C-3 and C-6, respectively. In the aliphatic region, one methine at H-9 appeared as a doublet at δ 3.60 (*J*=6.1 Hz). Three aliphatic methylene were observed at δ 3.32 (*dd*, *J*=6.4, 17.2 Hz) and δ 2.98 (*dd*, *J*= 6.4, 17.2 Hz) corresponding to H-10β and H-10α); and the signal at δ 2.46 *m* was assigned to H_2-16; and the signal at δ 1.82 *m* belongs to (H_2-15). The remaining signal at δ 2.40 *s*, indicated the presence of N-CH_3 (Blanchfield *et al.,* 2003; Chang *et al.,* 2001).

The ^{13}C-NMR spectrum (Figure 4.118**)** of **22** showed the presence of twenty carbon resonances, which is in agreement with the molecular formula $C_{20}H_{23}NO_4$. The characteristic signals of morphinandienone alkaloid of **22** was supported by the two significant signals, sp^3 quaternary carbon signal at C-13 and sp^2 conjugated carbonyl carbon at C-7 resonated at δ 41.1 and δ 180.3 respectively (Mukhtar *et al*., 2004b). The spectroscopic data of alkaloid **22** were identical with those reported for (+)sebiferine

previously isolated from the Lauraceae species (Bhakuni & Singh, 1979; Tackie *et al*., 1974; Lajis *et al.,* 1989).

Due to the limitation of the sample amount, we were unable to get its 2D-NMR spectrum such as COSY, HMQC and HMBC.

The ^{1}H and ^{13}C NMR data suggested that the compound to have morphinandienone skeleton and the most probable structure is N-methyl-2,3,6 trimethoxymorphinandien-7-one (Roblot *et al*., 1984). Therefore, on the basis of the above data analysis and comparison with literature values (Hara *et al*., 1995; Gözler *et al*., 1990; Wu *et al*., 1993; Bhakuni & Singh, 1979; Tojo *et al*., 1989; Mollataghi, 2012) the compound was deduced as sebiferine (**22**) or N-methyl-2,3,6 trimethoxymorphinandien-7-one.

Table 4.17: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of sebiferine (**22**) and the literature data.

	22 in $CDCl_3$		* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	6.58 (*s*, 1H)	110.0	6.60 (*s*, 1H)	110.5
2	-	148.5	-	148.2
3	-	148.7	-	148.6
4	6.77 (*s*, 1H)	108.3	6.78 (*s*, 1H)	108.6
5	6.32 (*s*, 1H)	118.6	6.33 (*s*, 1H)	118.5
6	-	151.7	-	151.6
7	-	180.3	-	180.3
8	6.22 (*s*, 1H)	122.1	6.31 (*s*, 1H)	124.1
9	3.60 (*d*, *J*=6.1)	60.2	3.61 (*d*, *J*=6.1)	61.1
10	β = 3.32 (*dd*, *J*=6.4, 17.2) α = 2.98 (*dd*, *J*=6.4, 17.2)	31.9	β = 3.33 (*d*, *J*=17.2) α = 3.01 (*dd*, *J*=6.6, 17.3)	31.0
11	-	128.1	-	128.1
12	-	129.4	-	130.0
13	-	41.1	-	42.0
14	-	161.5	-	151.6
15	2.82 (*m*, 2H)	40.6	2.58 (*m*, 2H)	45.6
16	2.46 (*m*, 2H)	45.1	2.45 (*m*, 2H)	40.1
2-OCH_3	3.82 (*s*, 3H)	55.8	3.82 (*s*, 3H)	56.3
3-OCH_3	3.78 (*s*, 3H)	55.5	3.70 (*s*, 3H)	55.9
6-OCH_3	3.65 (*s*, 3H)	55.4	3.85 (*s*, 3H)	55.2
N-Me	2.40 (*s*, 3H)	41.7	2.29 (*s*, 3H)	41.4

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Mollataghi, 2012).

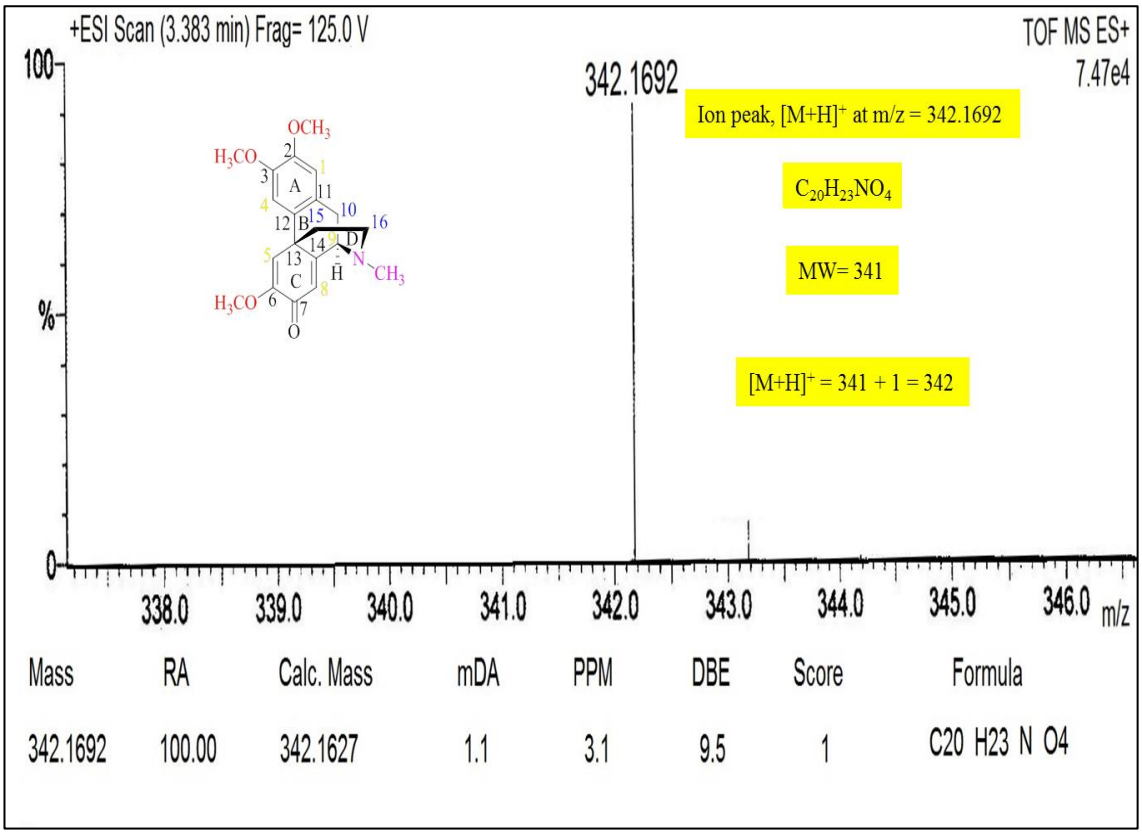


Figure 4.115: LCMS-ESI spectrum of sebiferine (**22**)

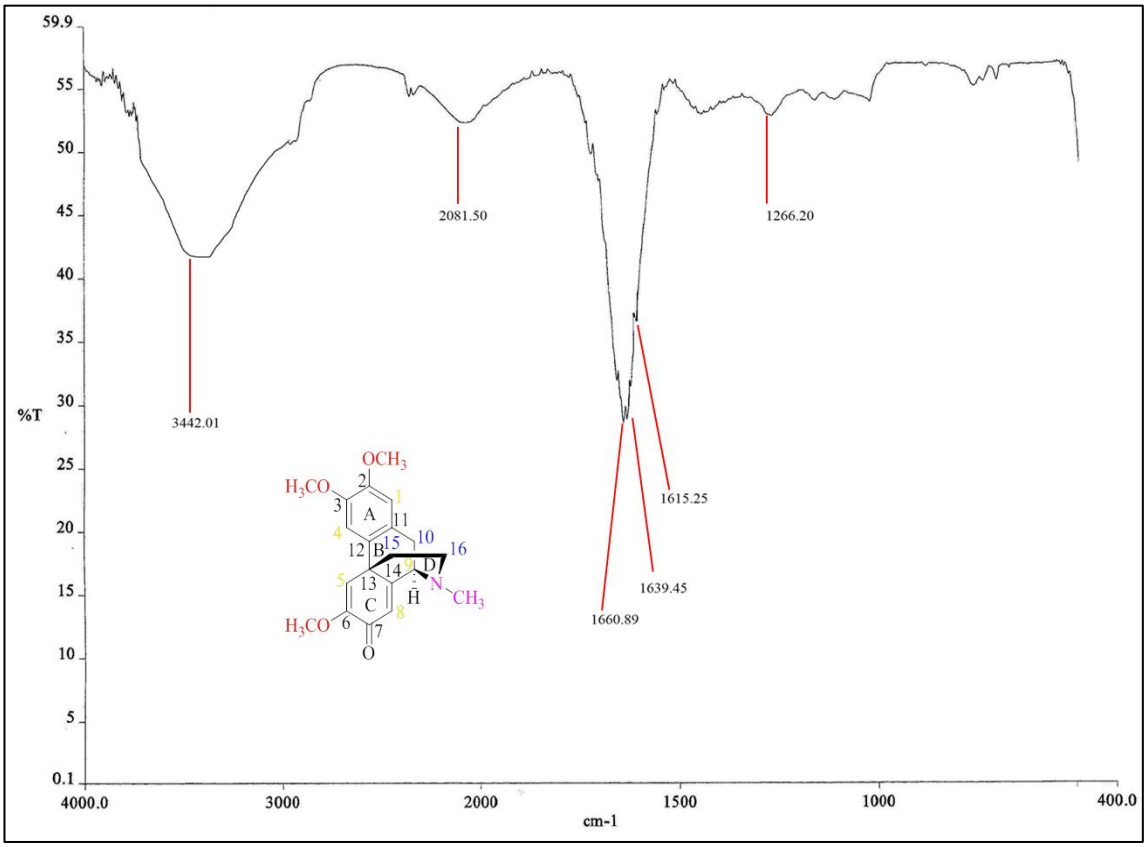


Figure 4.116: IR spectrum of sebiferine (**22**).

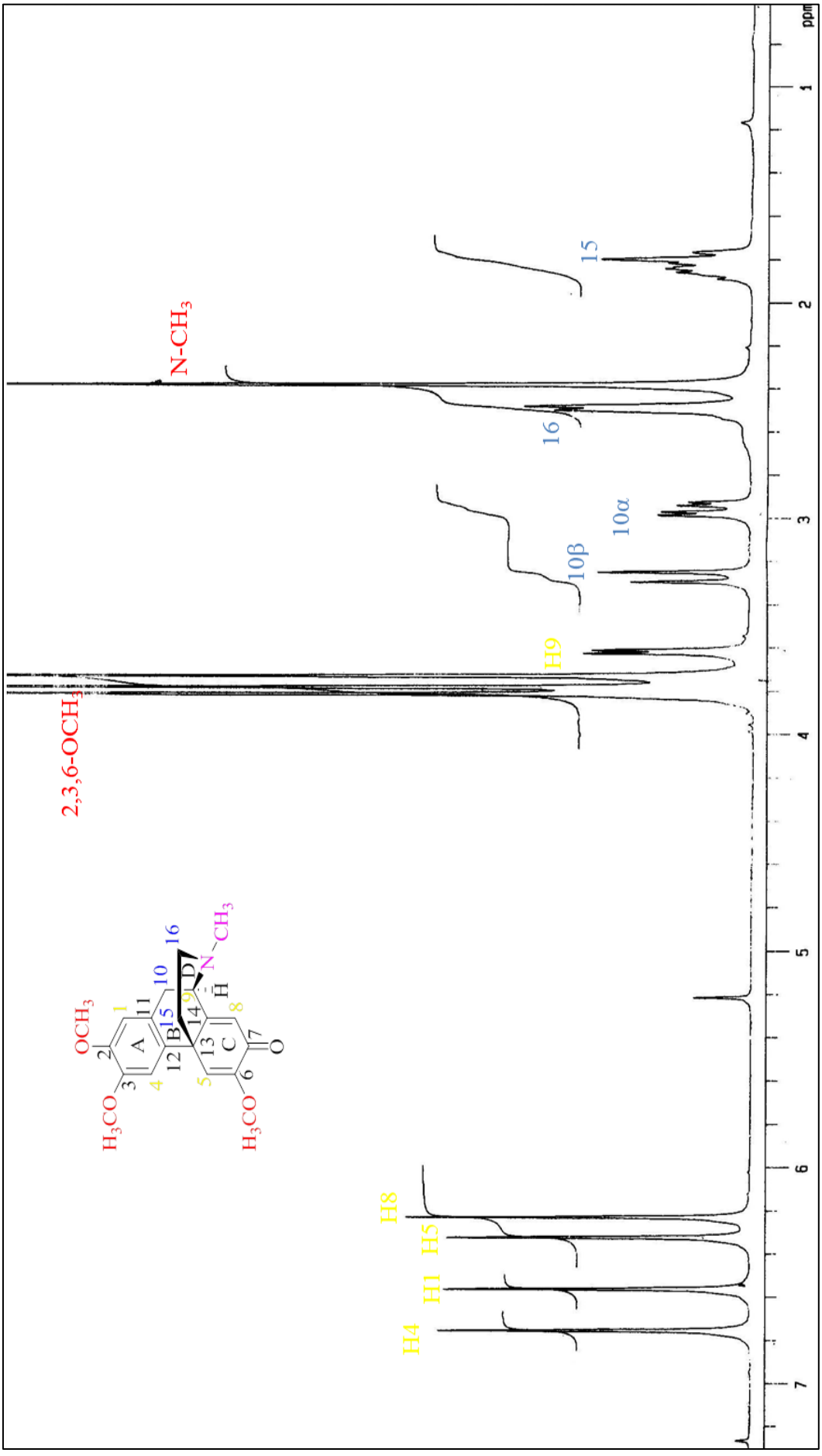


Figure 4.117: ^{1}H- NMR spectrum of sebiferine (**22**).

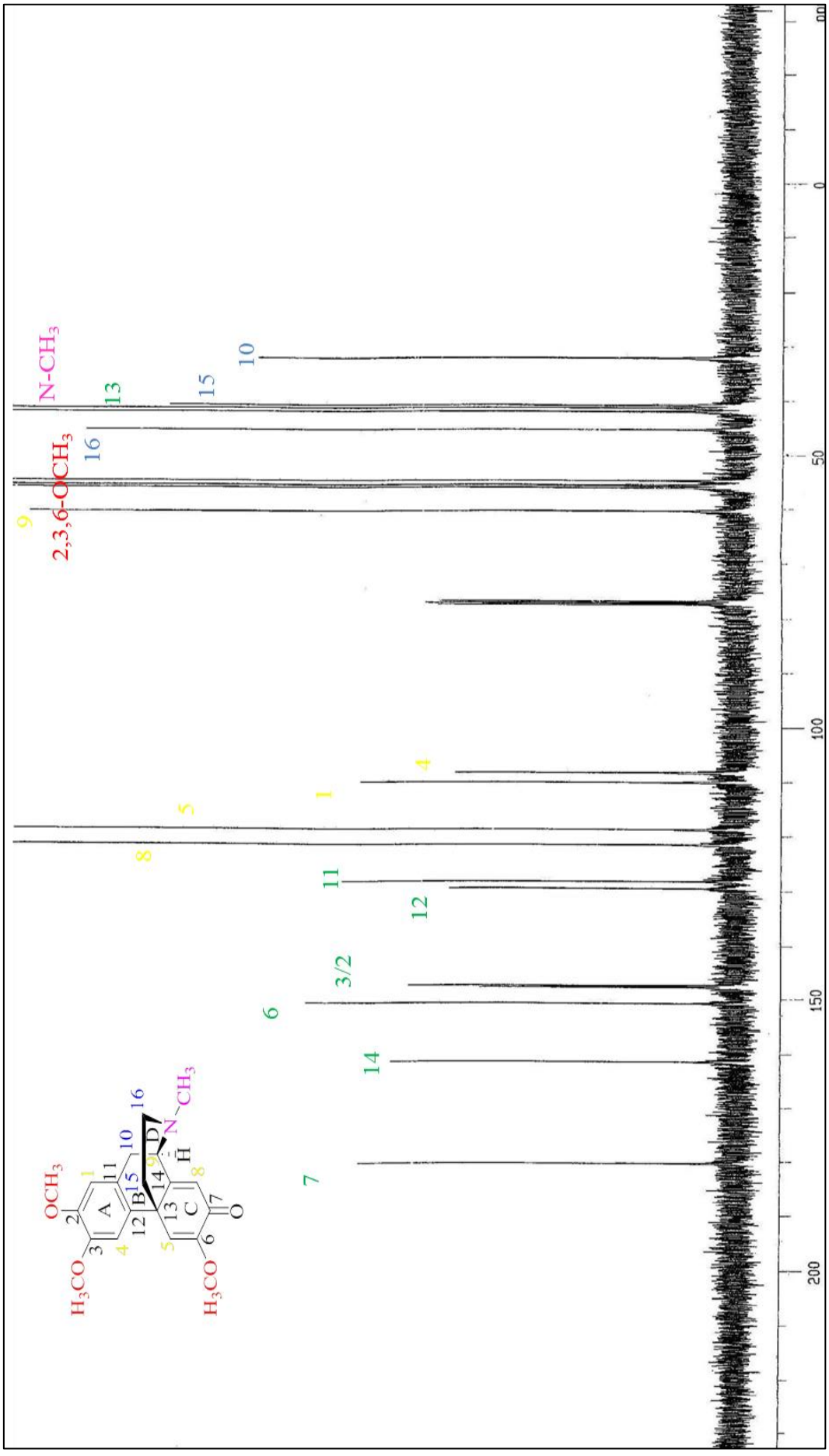


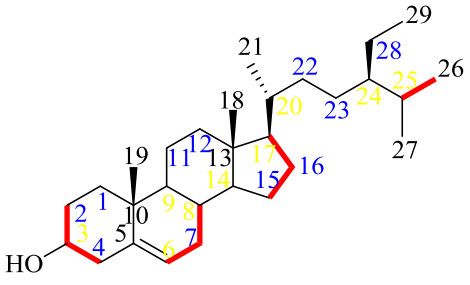
Figure 4.118: ^{13}C NMR spectrum of sebiferine (**22**).

4.5 Compounds isolated from the leaves of *Actinodaphne sesquipedalis* (HIR 011).

The isolation of phytochemicals from the leaves of *Actinodaphne sesquipedalis* (HIR 011) afforded four known compounds from the hexane extract which consist of β-sitosterol (**59**), dicentrine (**52**), N-methyllaurotetanine (**51**) and stigmasterol (**60**).

Further investigation on the MeOH extract of the leaves of *Actinodaphne sesquipedalis* (HIR 011) species led to the isolation one sterol compound, β-sitosterol (**59**) and together with five known aporphine alkaloids; namely dicentrine (**52**), dicentrinone (**58**), boldine (**5**), norisocorydine (**65**) and laurolitsine (**6**) or norboldine.

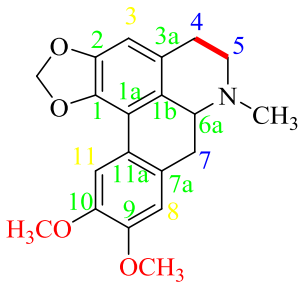
4.5.1 β-sitosterol (59)



59

Compound **59** with $[\alpha]^{25}_{D}$ = -36.6˚ (c= 0.15, $CHCl_3$) was isolated from hexane extract and methanol extract of the plant. The MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) data spectra features were assemble to β-sitosterol (**59**) isolated from hexane extract of bark of *Phoebe grandis*. In fact this compound is β-sitosterol (**59**) and has been discussed in Section **4.3.1** β-sitosterol, **59.** Please refer (page 156-168).

4.5.2 Dicentrine (52)



52

The major alkaloid **52** isolated from the leaves of *Actinodaphne sesquipedalis* crystallized as a colourless prism with the melting point 167-169°C. The LCMS-QTOF mass spectrum (Figure 4.119) showed a molecular ion peak at m/z = 340.1534 $[M+H]^+$, consistent with the molecular formula of $C_{20}H_{21}NO_4$ (calcd for $C_{20}H_{22}NO_4$, 340.1471). The UV spectrum of **52** exhibited peaks at 219, 281 and 305 nm which were consistent and supported the literature values at peaks 221, 281 and 306 nm (Zhou *et al*., 2000). Its UV spectrum also suggesting a 1,2,9,10-tetrasubstituted aporphine skeleton (Goodwin *et al.*, 1958).

Its IR spectrum (Figure 4.120) showed strong absorptions at 2916 cm^{-1} and 2790 cm^{-1} due to the stretching of C-H aromatic, respectively. The range of absorptions from 1344 – 1606 cm^{-1} indicated aromatic C=C stretching. The absorption at 1268 cm^{-1} indicated the presence of the methoxyl groups. While, the strong absorption peaks appeared at δ 1095 cm^{-1} and 940 cm^{-1} gave the characteristic of the presence of methylenedioxy group at C1 and C2. There is no signal peak of C=O carbonyl group at the range of 1700 cm^{-1} and no broad band of OH, hydroxyl stretching absorption signal at the range of 3000 - 3500 cm^{-1}. These data, proposed the structure of **52** as aporphine skeleton without carbonyl and hydroxyl group.

The proton NMR spectrum (Figure 4.121) indicated the presence of three isolated aromatic protons at δ 7.63, 6.75 and 6.48 assigned to H-11, H-8 and H-3 respectively of an aporphine alkaloid. Two doublets of two protons at δ 6.04 (*d, J*=1.3 Hz) and δ 5.89 (*d, J*=1.3 Hz) were consistent with a methylenedioxy substituent on C1-C2. Two singlets very close to each other at δ 3.88 and δ 3.87, attributed to two methoxyls attached to C-9 and C-10. The *N*-methyl protons appeared as singlet peak at δ 2.53 and integrated for three protons. A multiplet of methine signal appeared at δ 3.14 was attributed to the one proton, H-6a. Three aliphatic methylene protons were observed at δ 3.06 (*dd, J*=14.4, 4.4, H-4β) and δ 2.59 (*dd, J*=14.4, 4.4, H-4α); δ 3.03 (*dd, J*=12.4, 4.8, H-5β) and δ 2.49 (*dd, J*=12.4, 4.8, H-5α); and δ 3.04 (*d, J*=4.3, H-7β) and δ 2.64 (*d, J*=4.3, H-7α).

The ^{13}C (Figure 4.122) and DEPT (Figure 4.123) experiments showed the presence of twenty carbon signals made up of three aliphatic methylenes, four methines, two methoxyl, one *N*-methyl protons, nine quaternary carbons and one methylenedioxy. The chemical shift of the methylenedioxy moiety was observed at δ_C 100.6. The two methoxyl signals appeared at δ_{C10} 56.1 and δ_{C9} 55.9.

In order to know the positions of the remaining substitutions, a COSY experiment was performed. The COSY spectrum (Figure 4.124) revealed that the proton at δ 3.06 (H-4β) correlated with the proton at δ 2.49 (H-5α); proton at δ 3.02 (H-5β) correlated with the proton at δ 2.59 (H-4α); and proton at δ 3.14 (H-6a) correlated with the proton at δ 2.64 (H-7α).

The ^{1}H and ^{13}C direct correlations were determined by using HSQC spectrum (Figure 4.125) and the results were supported by other data. The placement of all protons and

quaternary carbons were confirmed by the ^{1}H-^{13}C long range correlations observed in the HMBC spectrum (Figure 4.126) of **52**.

Since the NMR assignments have not previously been reported completely in the literature, we assigned the ^{1}H and ^{13}C NMR spectra data unambiguously by the long range correlations in its HMBC, HSQC and COSY spectra as shown in Table 4.18.

Based on its spectral data and comparison with those of literature (Zhou *et al.,* 2000), the structure of compound was elucidated as dicentrine (**52**). Interestingly, it was isolated for the first time from leaves of *Actinodaphne sesquipedalis* but previously, dicentrine (**52**) had been isolated from the bark of *Actinodaphne sesquipedalis* (Laili Din, *et al*., 1994).

Table 4.18: ^{1}H-NMR (600 MHz),^{13}C-NMR (150 MHz) and 2D (HMBC and HSQC) NMR data of dicentrine (**52**) in $CDCl_3$ and the literature data.

	52 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^{2}J,^{3}J)	HSQC (^{1}J)	δ_H (ppm, *J* in Hz)	δ_C (ppm)
1	-	141.8	-	-	-	141.8
1a	-	116.6	-	-	-	116.6
1b	-	126.2	-	-	-	126.3
2	-	147.7	-	-	-	147.7
3	6.48 (1H, *s*)	106.8	C_{1b}, C_1, C_2, C_4	H_3	6.51 (1H, *s*)	106.8
3a	-	126.6	-	-	-	126.5
4β	3.06 (*dd*, *J*=14.4,4.4)	29.0	$C_{3.}$	$H_{4β}$	3.19 (*dd*, *J*=14.3,4.4)	29.0
4α	2.59 (*dd*, *J*=14.4, 4.4)		$C_{3.}$	$H_{4α}$	2.66 (*dd*, *J*=14.3, 4.3)	
5β	3.03 (*dd*, *J*=12.4, 4.8)	53.5	C_4, $C_{6a,}$ N-$CH_{3.}$	$H_{5β}$	2.54 (*dd*, *J*=11.5,11.9)	53.5
5α	2.49 (*dd*, *J*=12.4, 4.8)		C_4, $C_{6a,}$ N-$CH_{3.}$	$H_{5α}$	2.54 (*dd*, *J*=11.5,11.9)	
6a	3.14 (1H, *m*)	62.3	C_5,C_{3a},C_7, N-CH_3	H_{6a}	3.10 (1H, *m*)	62.3
7β	$H_{7β}$ =3.04 (d, *J*=4.3)	34.1	C_{11}, C_8, $C_{1b.}$	$H_{7β}$	$H_{7β}$ =3.08 (1H, *m*)	34.1
7α	$H_{7α}$ =2.64 (*d*, *J*=4.3)		C_{11}, C_8, $C_{1b.}$	$H_{7α}$	$H_{7α}$ =2.63 (1H, *m*)	
7a	-	128.3	-	-	-	128.2
8	6.75 (1H, *s*)	111.3	C_{11a}, $C_{9,}$ C_7	H_8	4.77 (1H, *s*)	111.2
9	-	148.3	-	-	-	148.2
10	-	146.7	-	-	-	146.7
11	7.63 (1H, *s*)	110.6	C_{1a}, C_{7a}, C_9	H_{11}	7.66 (1H, *s*)	110.5
11a	-	123.6	-	-	-	123.5
9-OCH_3	3.88 (3H, *s*)	55.9	C_8, C_{10}	$3H_{9-OMe}$	3.92 (3H, *s*)	55.9
10-OCH_3	3.87 (3H, *s*)	56.1	C_{11}, C_9	$3H_{10-OMe}$	3.91 (3H, *s*)	56.1
N-CH_3	2.53 (3H, *s*)	43.7			2.56 (3H, *s*)	43.7
OCH_2O	6.04 (1H, *d*, *J*=1.32) 5.89 (1H, *d*, *J*=1.32)	100.6	C_1, C_2	$2H_{OCH2O}$	6.07 (1H, *d*, *J*=1.5) 5.92 (1H, *d*, *J*=1.5)	100.6

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Zhou *et al*., 2000).

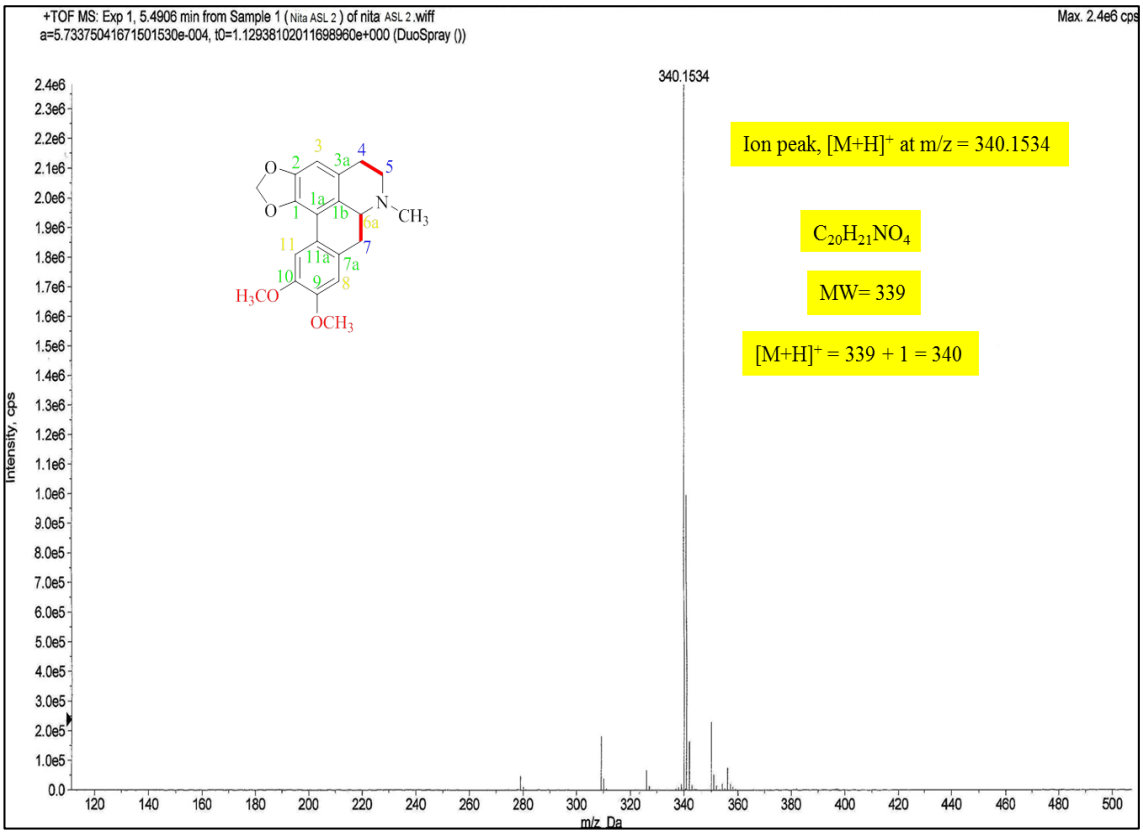


Figure 4.119: LCMS-QTOF spectrum of dicentrine (**52**).

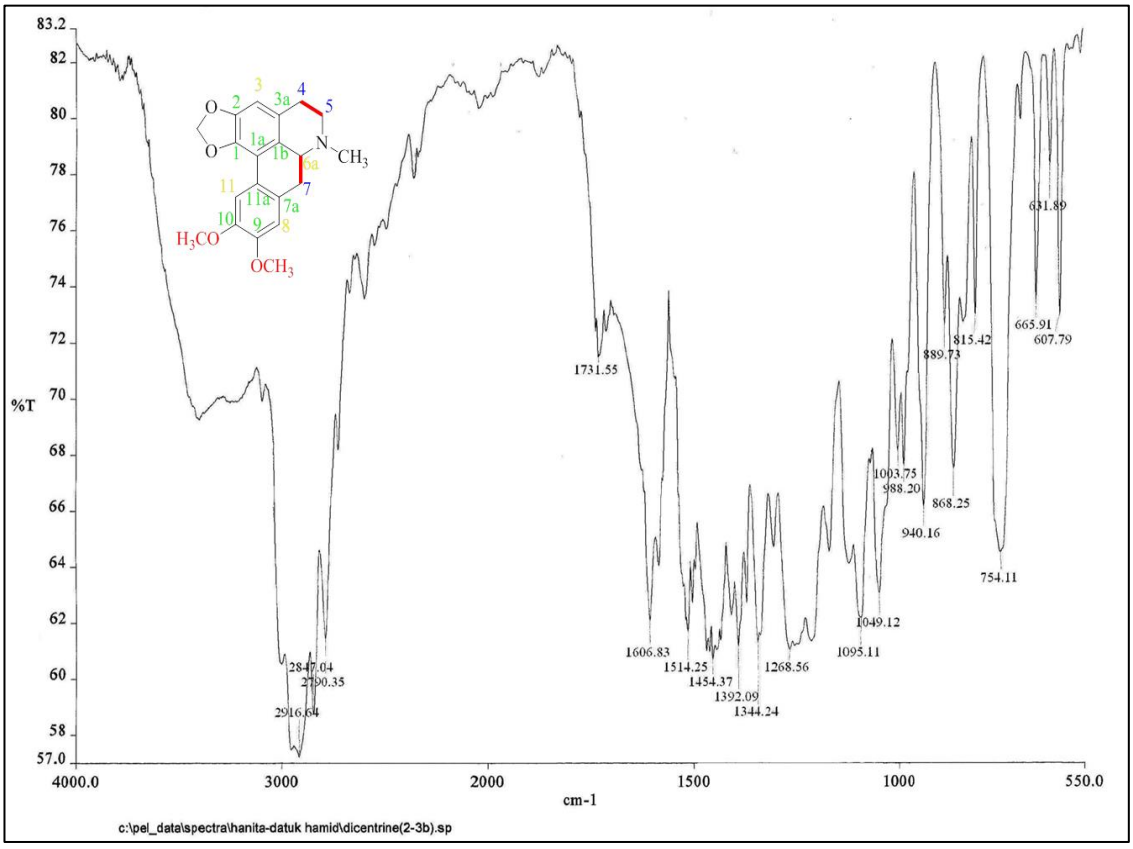


Figure 4.120: IR spectrum of dicentrine (**52**).

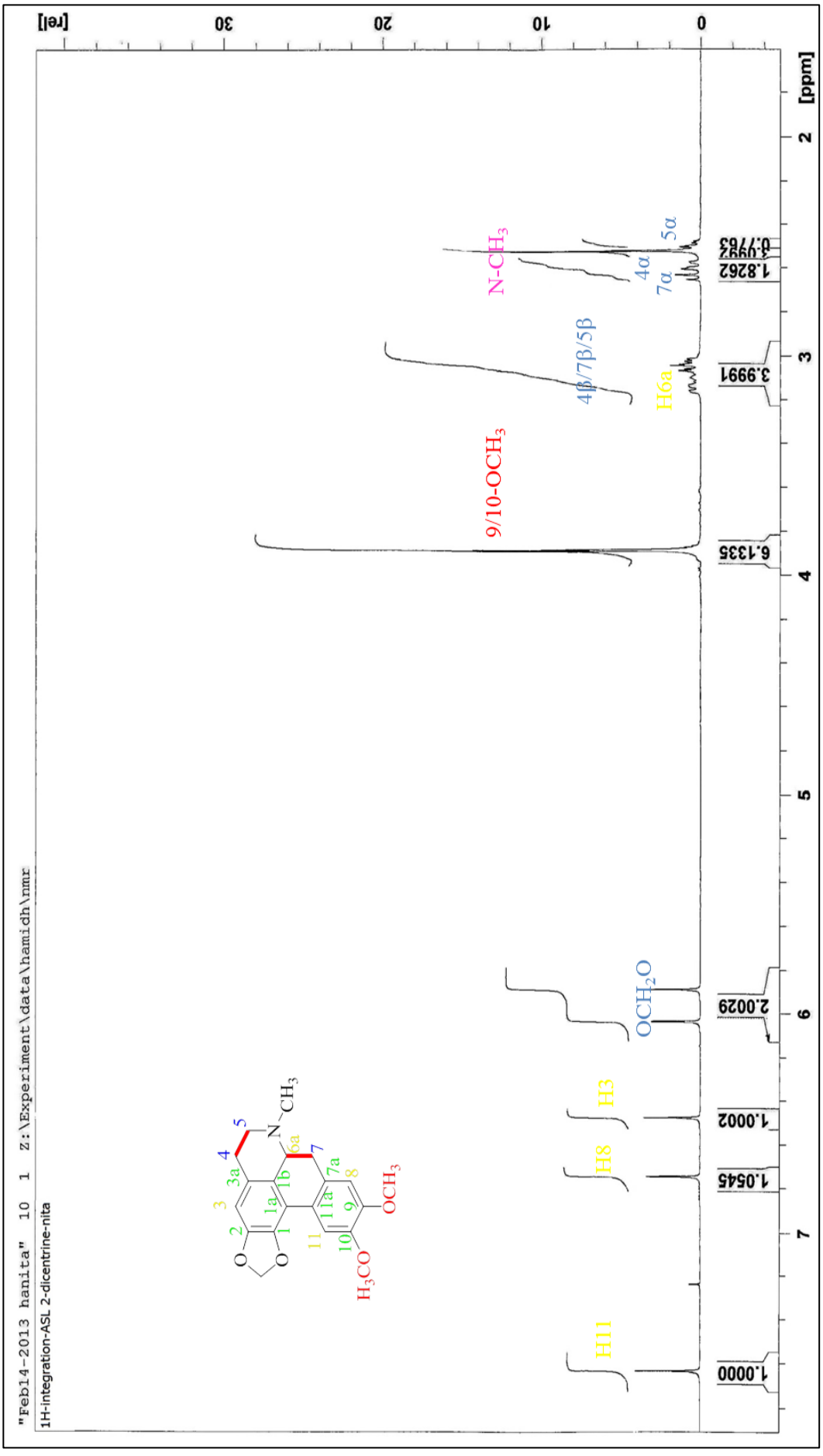


Figure 4.121: ^{1}H- NMR spectrum (integration) of dicentrine (**52**)

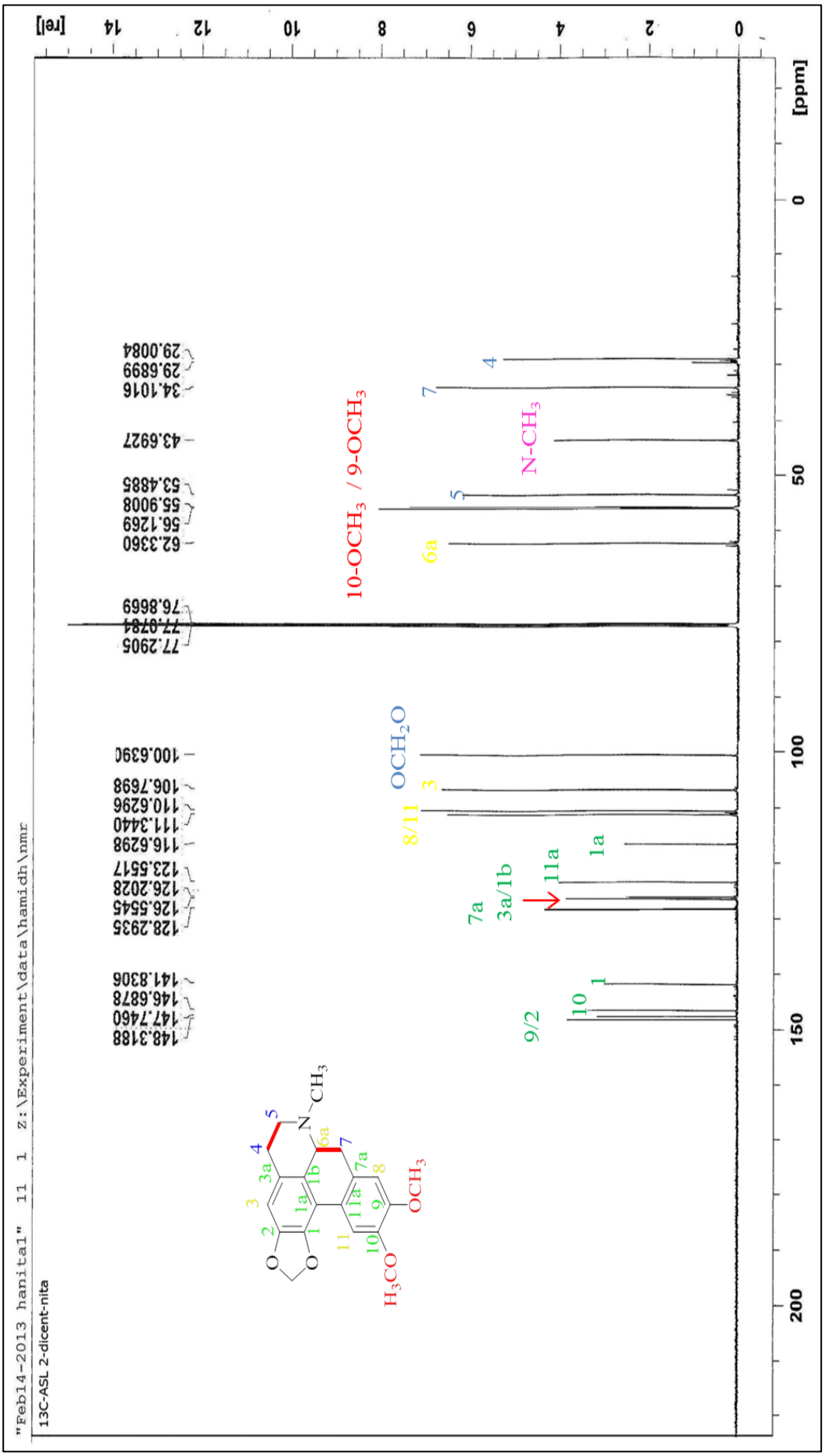


Figure 4.122: ^{13}C NMR spectrum of dicentrine (**52**)

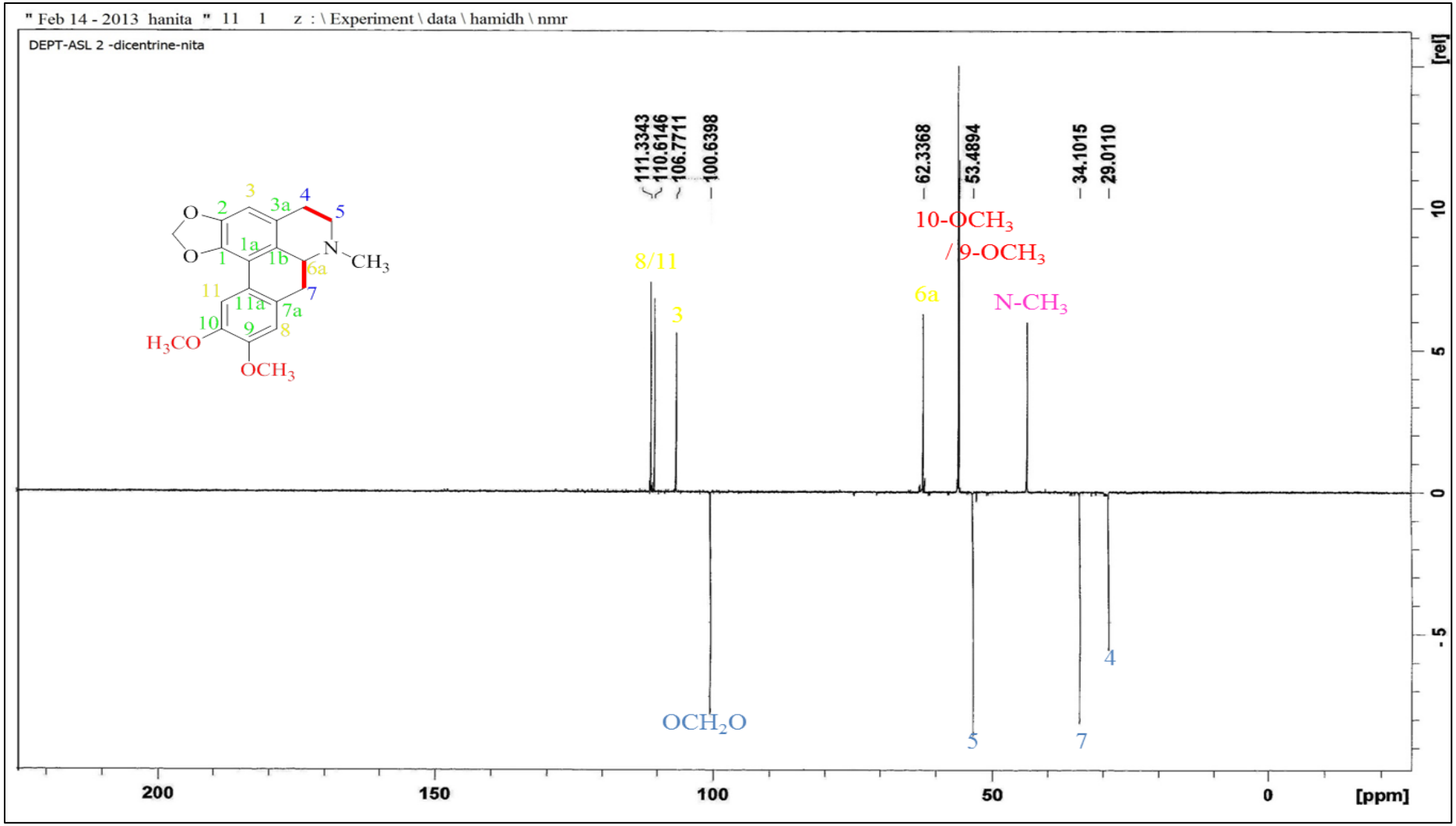


Figure 4.123: DEPT 135- NMR spectrum of dicentrine (**52**)

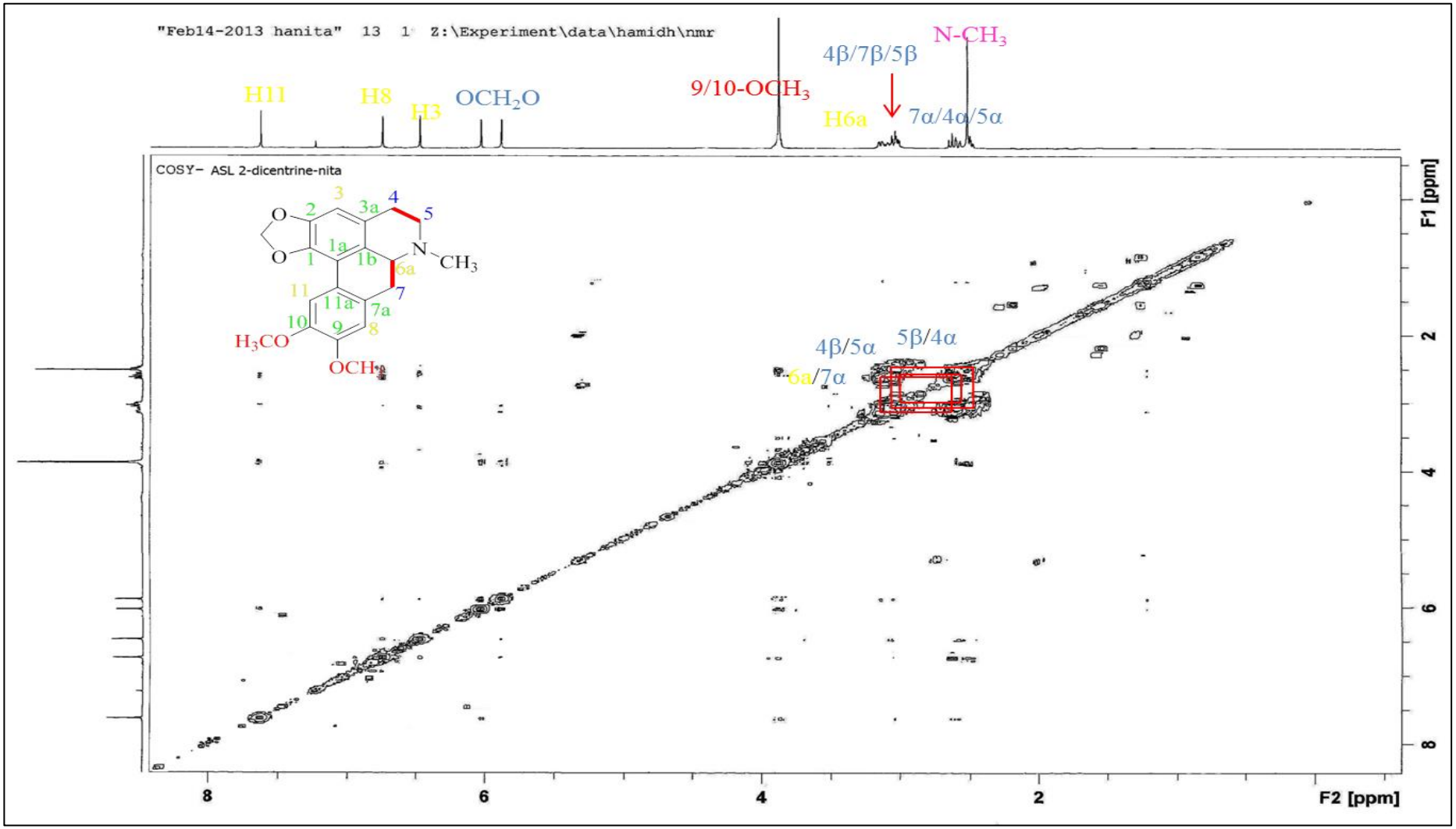


Figure 4.124: ^{1}H-^{1}H COSY- NMR spectrum of dicentrine (**52**)

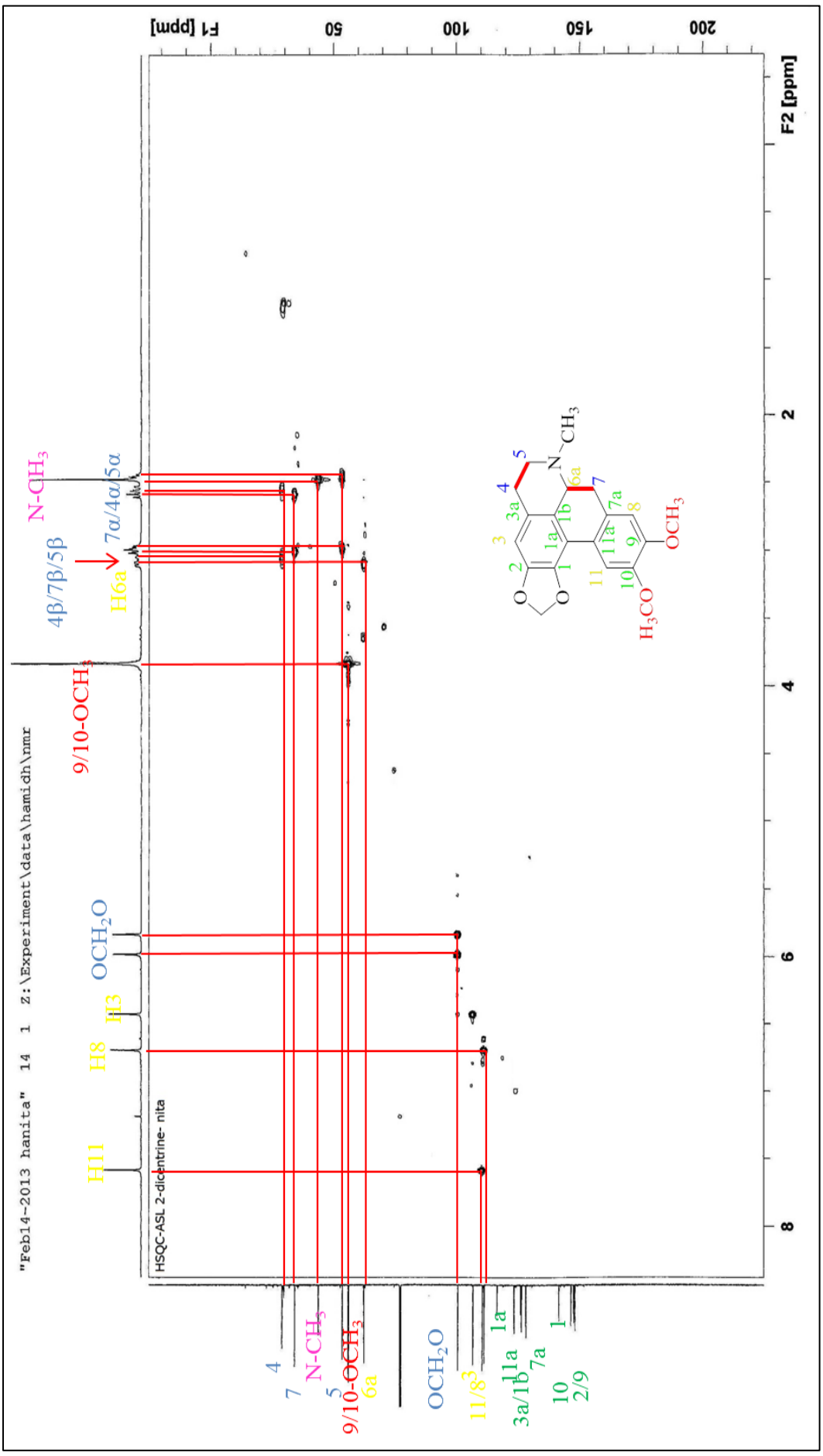


Figure 4.125: ^{1}H- ^{13}C HSQC- NMR spectrum of dicentrine (**52**)

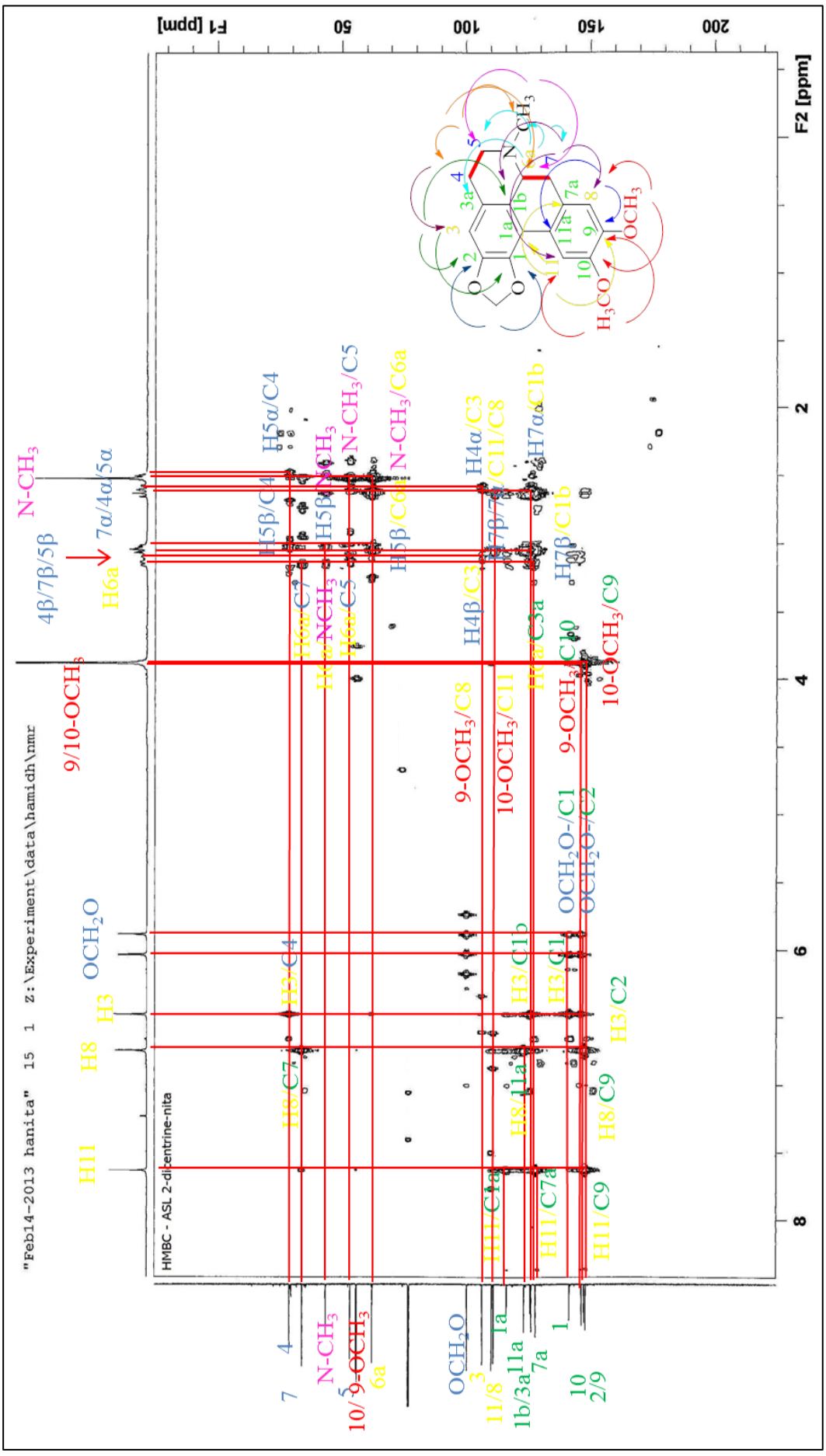
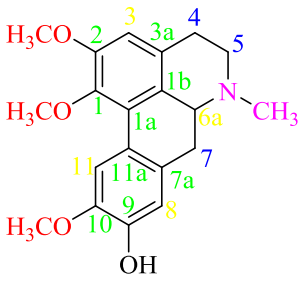


Figure 4.126: ^{1}H- ^{13}C HMBC- NMR spectrum of dicentrine (**52**)

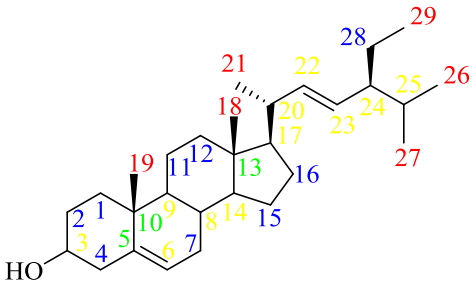
4.5.3 N-methyllaurotetanine (51)



51

Alkaloid **51** was obtained as a yellow amorphous solid with $[\alpha]_D^{25}$ = +111 (c=0.86, MeOH). The UV spectrum exhibit maxima at 215, 283 and 305 nm eliminated the possibility of a 9,10-disubstitution. The IR spectrum showed absorption peak at 3390 and 2935 cm^{-1} indicating a broad band of hydroxyl and the presence of CH aromatic, respectively. The MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) spectral data were identical to N-methyllaurotetanine (**51**) isolated from dichloromethane extract of bark of *Phoebe grandis*, previously discussed in section **4.3.4** (page 183-193).

4.5.4 Stigmasterol (60)

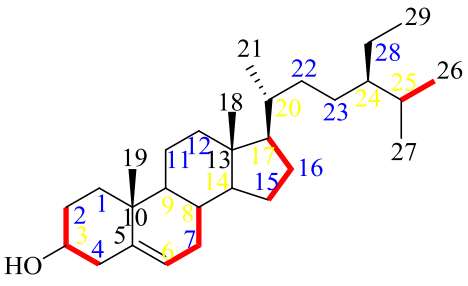


60

Compound **60** was isolated from the hexane extract of the leaves of *Actinodaphne sesquipedalis*. The MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) spectral data were identical

to stigmasterol (**60**) isolated from hexane extract of bark of *Phoebe grandis*, previously discussed in section **4.3.2** (refer page 169-175).

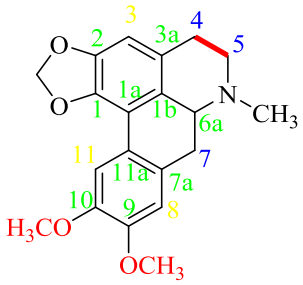
4.5.5 β-sitosterol (59)



59

Compound **59** with $[\alpha]^{25}_{D}$ = -36.6˚ (c= 0.15, $CHCl_3$) was also isolated from hexane extract and methanol extract of leaves of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) spectral data were identical to β-sitosterol (**59**) isolated from hexane extract of bark of *Phoebe grandis*, previously discussed in section **4.3.1** (refer page 156-168).

4.5.6 Dicentrine (52)

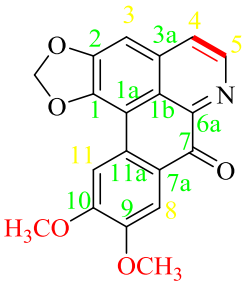


52

This major alkaloid **52** also isolated from the methanol extract of leaves of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC and HSQC) spectral data were identical to dicentrine (**52**)

that previously isolated from hexane extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **4.5.2** (refer page 262-272).

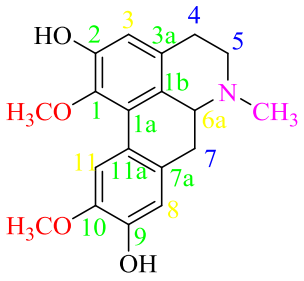
4.5.7 Dicentrinone (58)



58

Compound **58** was obtained as fine yellow needles. The UV, IR, MS, 1D-NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D-NMR (COSY, HMBC and HSQC) data spectra features were resemble to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis*. In fact dicentrinone (**58**) has been discussed previously in section **4.2.5** (refer page 147-155).

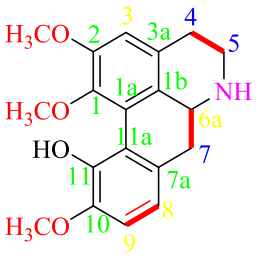
4.5.8 Boldine (5)



5

Alkaloid **5** was isolated as a brownish amorphous solid. The UV, IR, 1D-NMR (^{1}H NMR and ^{13}C NMR) data spectra and NOE different experiment features were similar to those of boldine (**5**) isolated from dichloromethane extract of bark of *Phoebe grandis*. It was described earlier in section **4.3.3** (refer page 176-182).

4.5.9 Norisocorydine (65)



65

The minor alkaloid **65** isolated from the leaves of *Actinodaphne sesquipedalis* as a brownish amorphous solid with the melting point 201-203°C and $[\alpha]_D^{25}$ = + 150.8° (c=0.08, CH_3OH). The molecular formula of **65** was deduced to be $C_{19}H_{21}NO_4$ based on the LCMS-ESI mass spectrometry (Figure 4.127), which gave a molecular ion peak at m/z 328.1538 $[M+H]^+$, (calcd. for $C_{19}H_{22}NO_4$, 328.1549). The UV spectrum showed an absorption bands at 223, 269 and 308 nm, thus suggesting a 1,2,9,10-tetrasubstituted aporphine skeleton (Nozaka *et al.*, 1990; Pyo *et al.*, 2003). In addition, the IR spectrum (Figure 4.128), gave a broad band between 3400 cm^{-1} and 2960 cm^{-1} due to the presence of OH and NH groups (Silverstein *et al.*, 1998; Duddley & Ian, 1989). The strong absorption appearing at 1285 cm^{-1} in the IR spectrum suggested the presence of the methoxyl groups, 2960 and 2850 cm^{-1} represent for CH-aromatic.

The ^1H NMR spectrum (Figure 4.129) revealed the characteristic of methoxyl group singlets at δ 3.90 (10-OCH_3), 3.89 (2-OCH_3) and 3.67 (1-OCH_3). One aromatic proton appeared as a singlet at δ 6.70 was attributable to H-3, indicating that C-1 and C-2 in ring A are disubstituted with methoxyl groups. In addition, H-9 and H-8 signals appeared as doublet of the vicinal protons at δ 6.85 and 6.75 respectively with the coupling constant of 8.2 Hz. The H-9 peak resided at the highest chemical shifts since there was a methoxyl group attached to the C-10. H-5 proton appeared at lower field

compared to H-4 due to the withdrawing effect of the neighbouring electronegative N-atom.

The ^{13}C NMR (Figure 4.130) gave a total of nineteen carbons which validated the molecular formula $C_{19}H_{21}NO_4$. Analysis from the ^{13}C NMR (Figure 4.130) and DEPT (Figure 4.131) assignments; indicated the presence of nine quaternary, three aliphatic methylene, three aromatic carbons, three methoxyl groups and one methine carbon. It showed three signals of methoxyl groups which appeared at δ 62.3 (1-OCH_3), 56.7 (2-OCH_3) and 56.5 (10-OCH_3). In addition, signals at δ 151.5, 149.5, 142.1 and 144.2 were consistent with the presence of four oxygenated aromatic quaternary carbons. The former three were assigned to three methoxyl carbons at C-2, C-10 and C-1, while the latter has to be attached to the hydroxyl carbon at C-11.

The assignment of the aromatic protons on ring D was also confirmed by analysis of the COSY data (Figure 4.132) which showed H-9 (δ 6.85) only correlated with H-8 (δ 6.75). The aliphatic protons H-5β showed an interaction with H-4β, while H-5α showed an interaction with H-4α. H-7 proton is coupled to H-6a. These match with the suggested structure.

The exact position of the methoxyl groups were confirmed by means of ^{1}H-^{1}H NOESY (Figure 4.133) and HMBC experiment (Figure 4.135). The ^{3}J interaction between methoxyl signals at δ 3.90 (10-OCH_3) and C-10 (δ149.5); δ 3.89 (2-OCH_3) and C-2 (δ 151.5); δ 3.67 (1-OCH_3) and C-1 (δ 142.1) in the HMBC spectrum, further confirmed the exact location of methoxyls at C-10, C-2 and C-1. The ^{1}H-^{13}C direct correlations were determined by using HSQC spectrum (Figure 4.134) further supported the above data.

Previously the NMR data were not reported completely and hence we undertook proper assignment the ^{1}H and ^{13}C NMR chemical shifts of both protons and carbons unambiguously based on correlations in HMBC, HSQC and COSY spectra as shown in Table 4.19.

Based on these spectra data, the structure for this alkaloid is assigned as norisocorydine (**65**) and the data were in full agreement with reported values (Chang *et al.*, 2001). Interestingly, the compound was isolated for the first time from this plant.

Table 4.19: ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) data and 2D (HMBC and HSQC) NMR data of norisocorydine (**65**) and the literature data.

	65 in $CDCl_3$				* in $CDCl_3$
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (ppm, *J* in Hz)
1	-	142.1	-	-	-
1a	-	125.8	-	-	-
1b	-	129.9	-	-	-
2	-	151.5	-	-	-
3	6.70 (*s*, 1H)	111.8	C_{1b}, C_4, C_1, C_2	H_3	6.90 (*s*, 1H)
3a	-	130.4	-	-	-
4β	3.00 (*m*, 1H)	29.4	C_5, $C_{1b,}$ C_3.	$2H_4$	
4α	2.70 (*m*, 1H)				
5β	3.43 (*m*, 1H)	42.9	C_{6a}, C_{3a}	$2H_5$	
5α	2.92 (*m*, 1H)				
6a	3.67 *m*	54.2	-	H_{6a}	3.63 (*d*, *J*=3.6)
7β	2.76 (*dd*, *J*=4.1, 13.3)	38.5	C_{6a}, C_8, C_{11a}, C_{1b}, $C_{11.}$	$2H_7$	
7α	2.56 (*t*, *J*=13.3)		C_{6a}, C_8, C_{11a}, C_{1b}, $C_{11.}$		
7a	-	130.6		-	-
8	6.75 (*d*, *J*=8.2)	119.3	C_7, C_{11a}, C_{10}	H_8	6.87 (*d*, *J*=8.4)
9	6.85 (*d*, *J*=8.2)	110.9	C_{7a},C_{11}	H_9	6.95 (*d*, *J*=8.4)
10	-	149.5	-	-	-
11	-	144.2	-	-	-
11a	-	120.2	-	-	-
1-OMe	3.67 (*s*, 3H)	62.3	C_1	$3H_{1\text{-OMe}}$	3.67 (*s*, 3H)
2-OMe	3.89 (*s*, 3H)	56.7	C_2	$3H_{2\text{-OMe}}$	3.90 (*s*, 3H)
10-OMe	3.90 (*s*, 3H)	56.5	C_{10}	$3H_{10\text{-OMe}}$	3.87 (*s*, 3H)

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Chang *et al*., 2001).

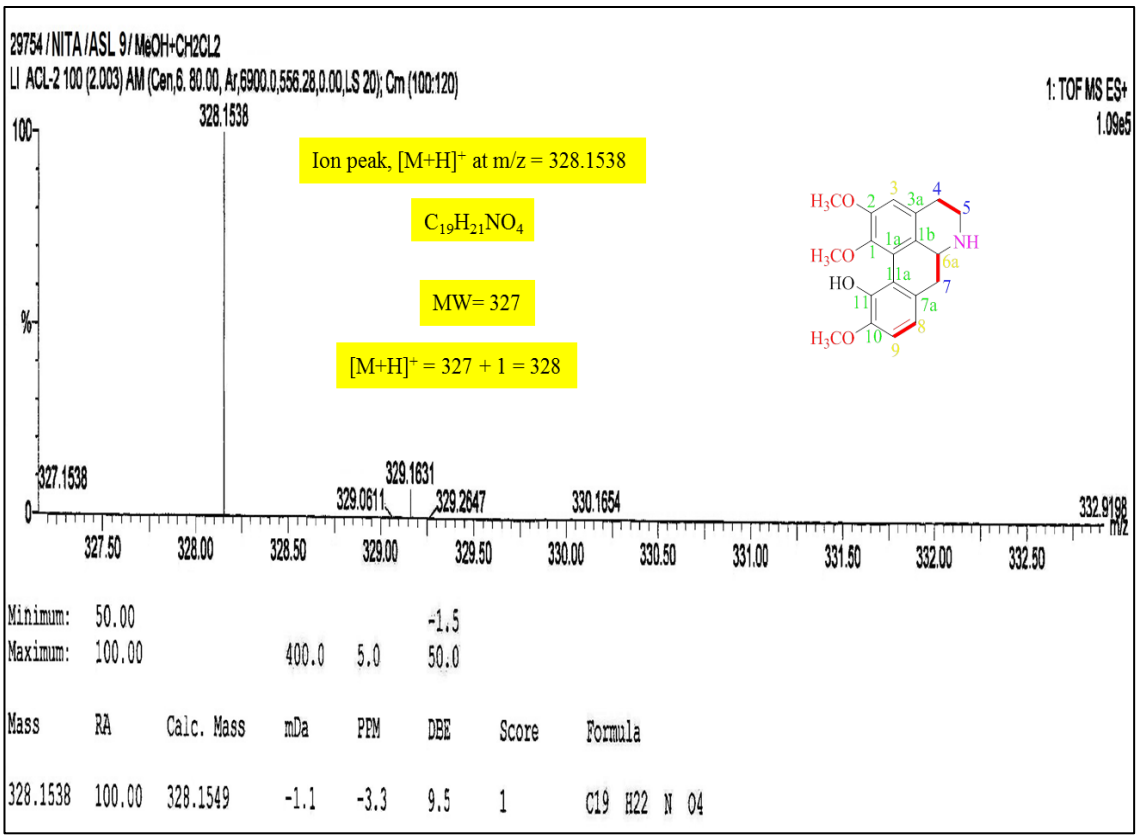


Figure 4.127: LCMS-ESI spectrum of norisocorydine (**65**).

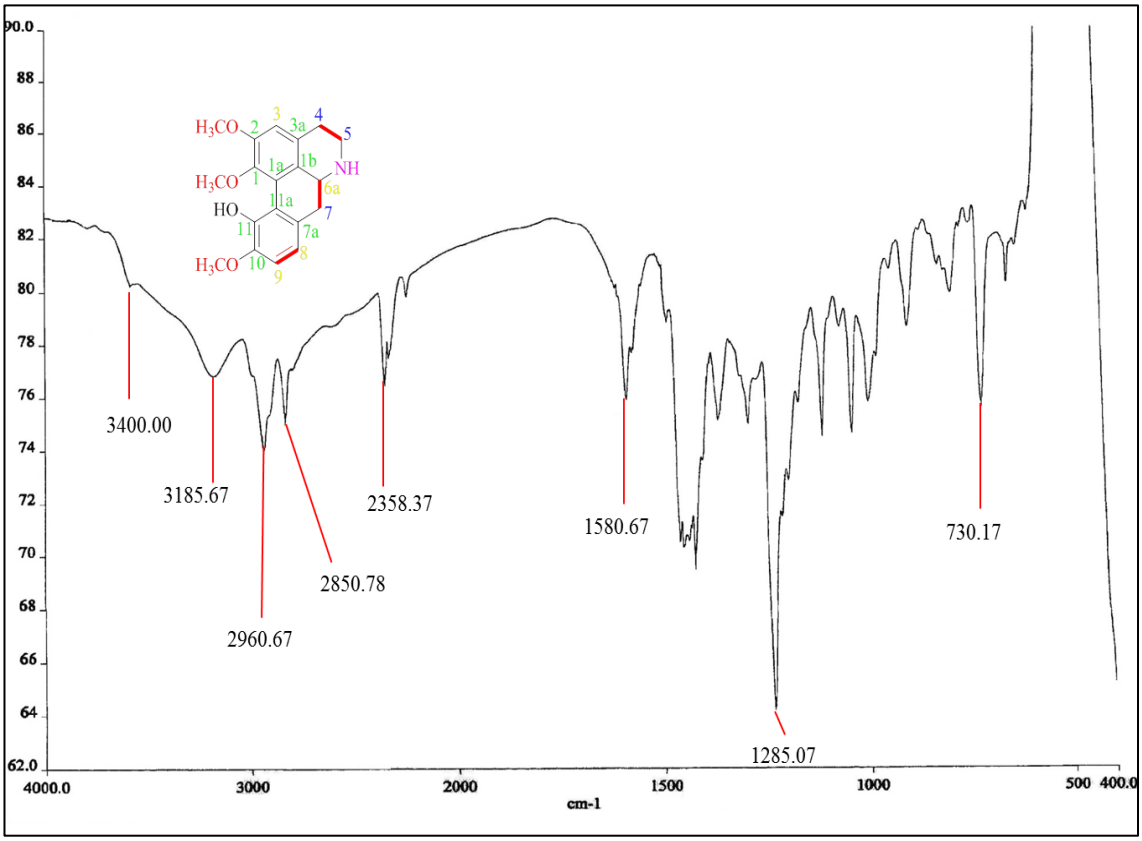


Figure 4.128: IR spectrum of norisocorydine (**65**).

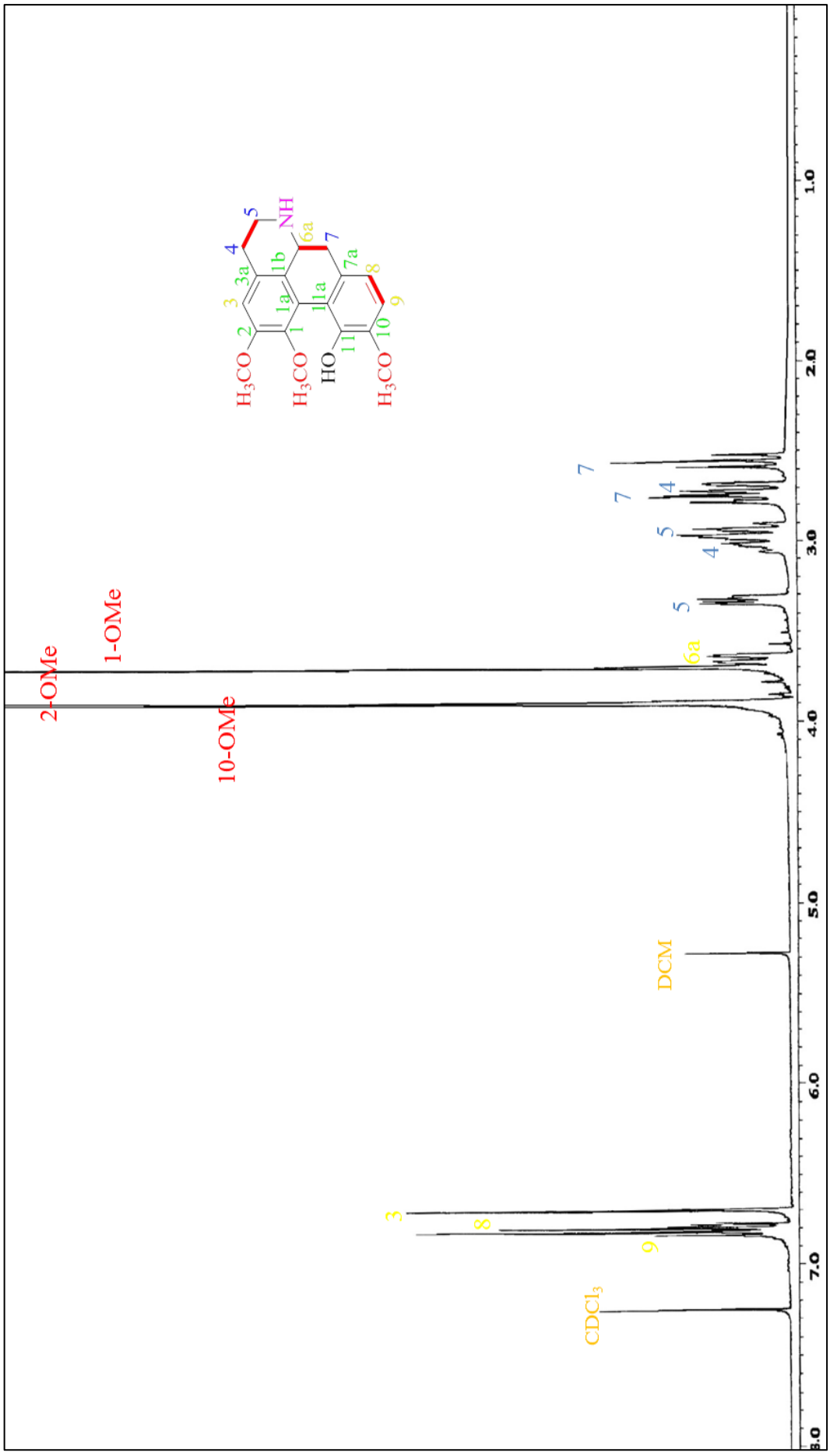


Figure 4.129: ^{1}H- NMR spectrum of norisocorydine (**65**).

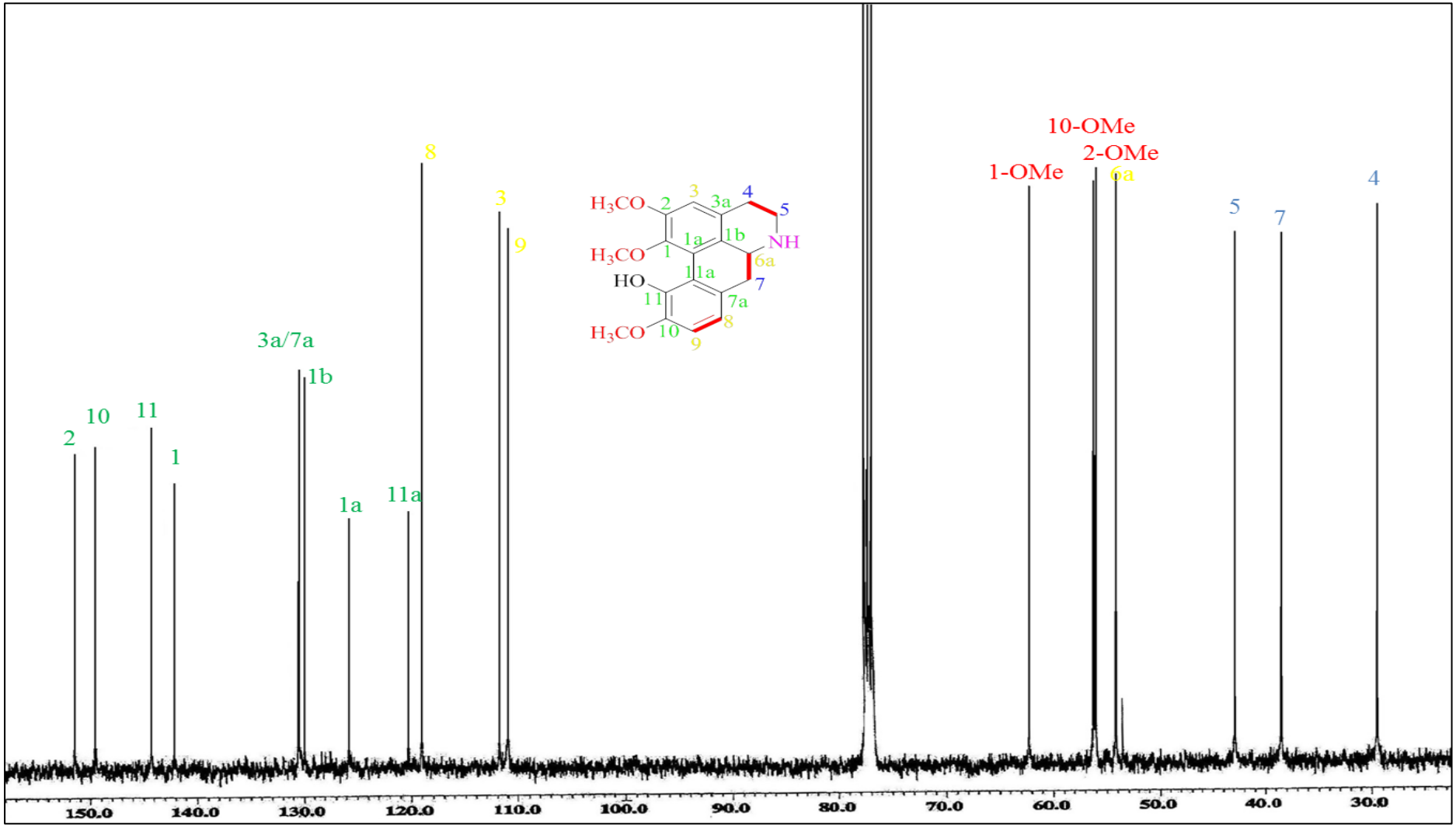


Figure 4.130: ^{13}C-NMR spectrum of norisocorydine (**65**).

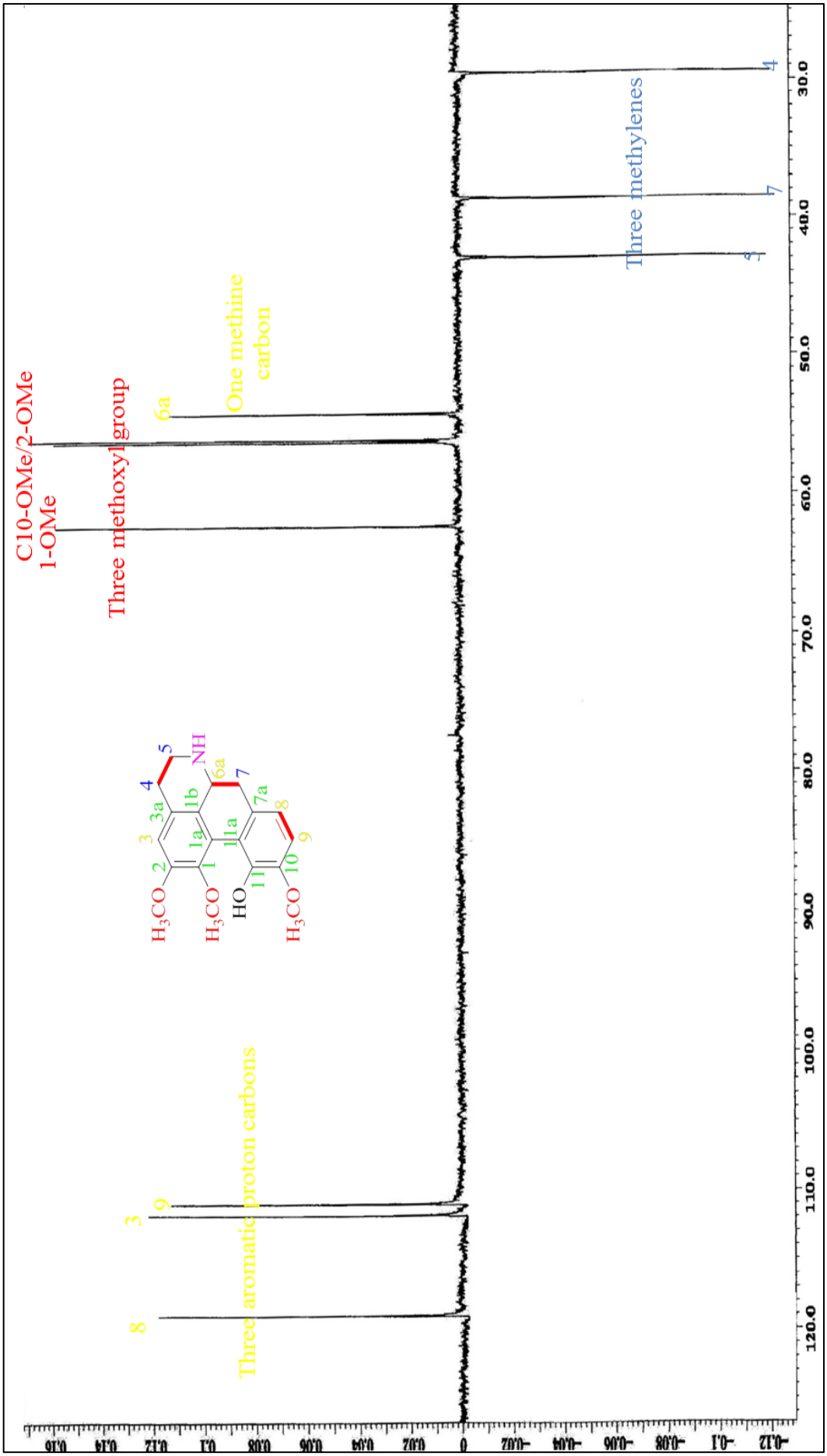


Figure 4.131: DEPT spectrum of norisocorydine (**65**).

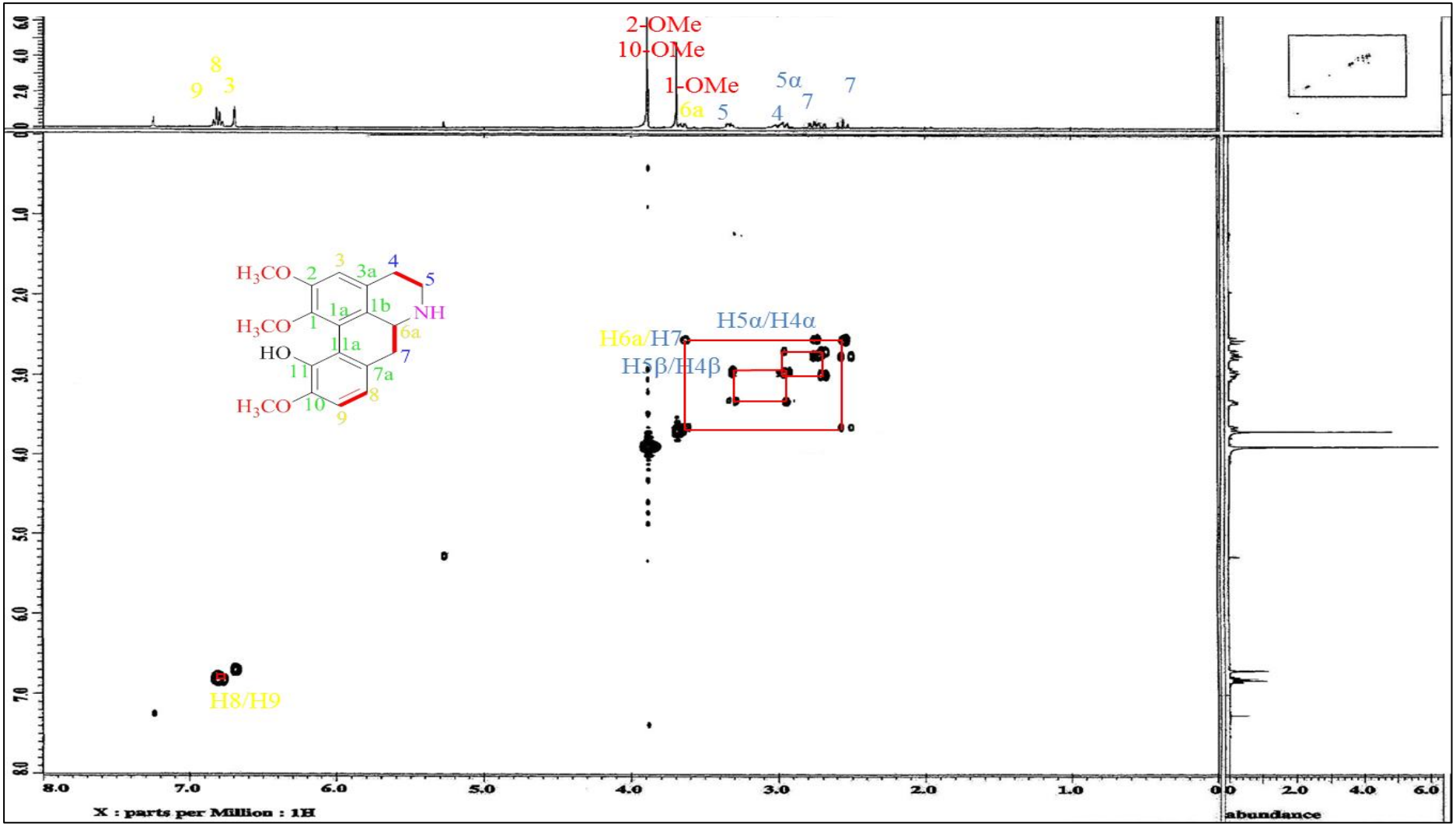


Figure 4.132: COSY spectrum of norisocorydine (**65**)

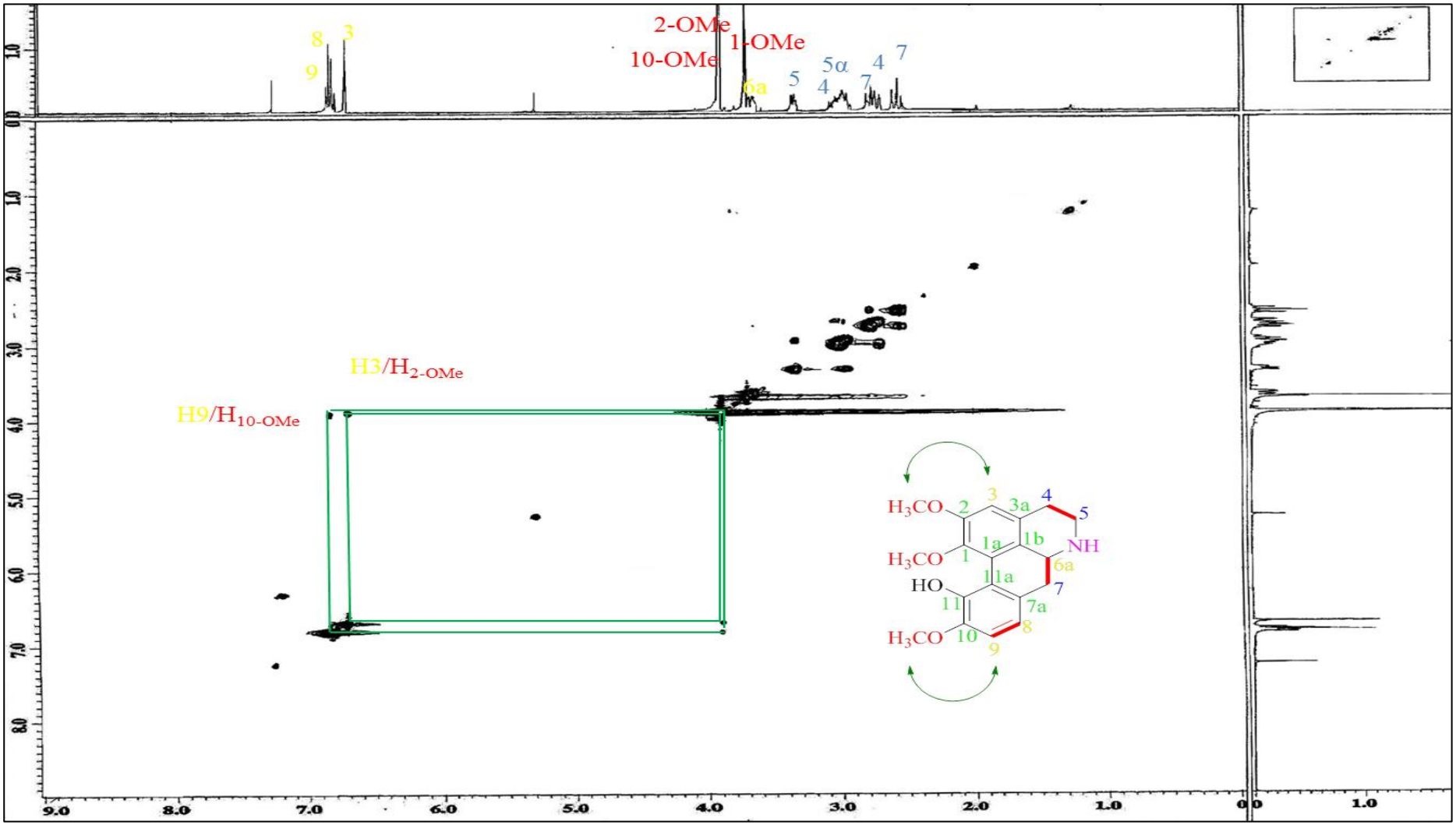


Figure 4.133: NOESY spectrum of norisocorydine (**65**).

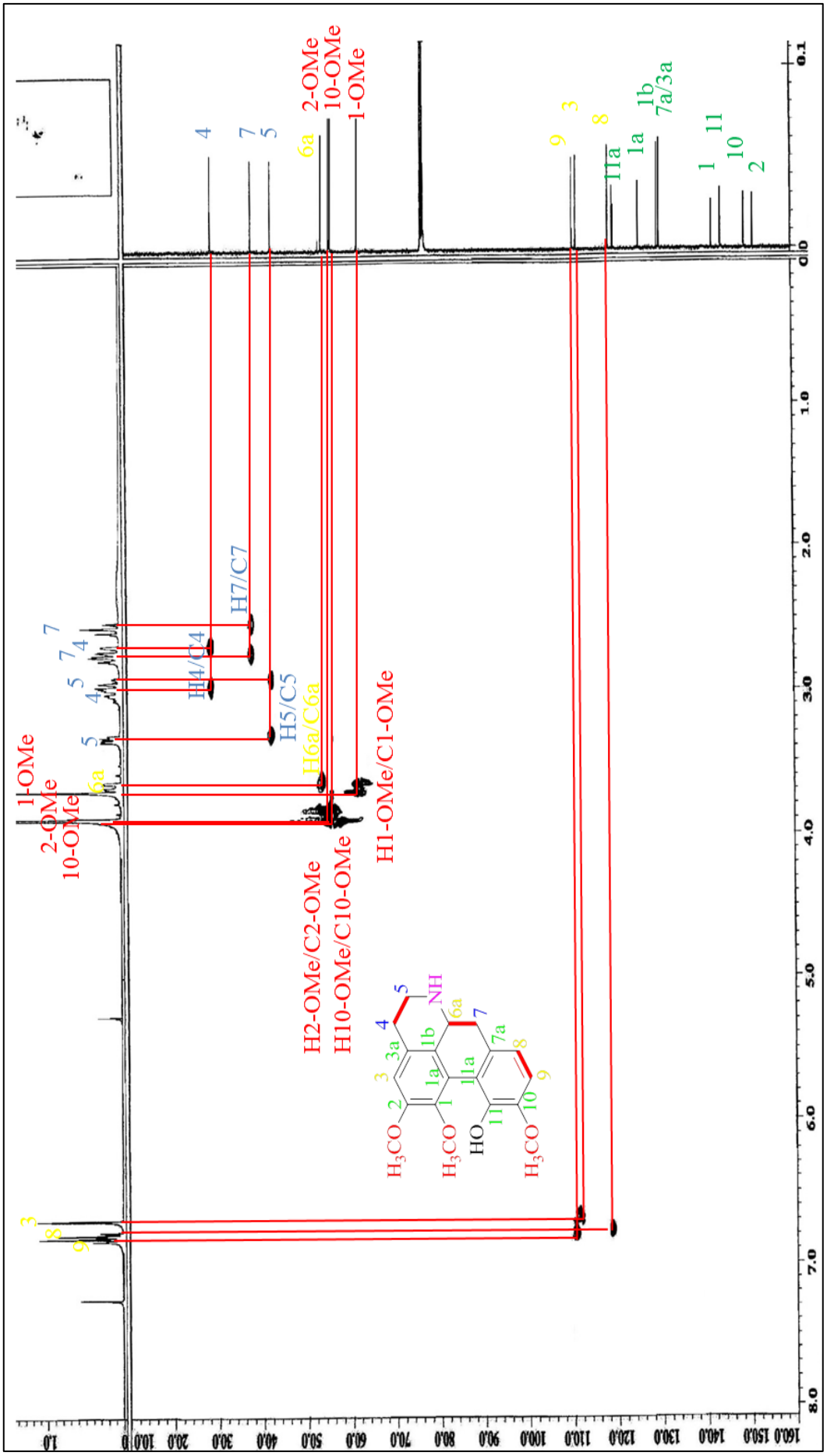


Figure 4.134: HSQC spectrum of norisocorydine (**65**).

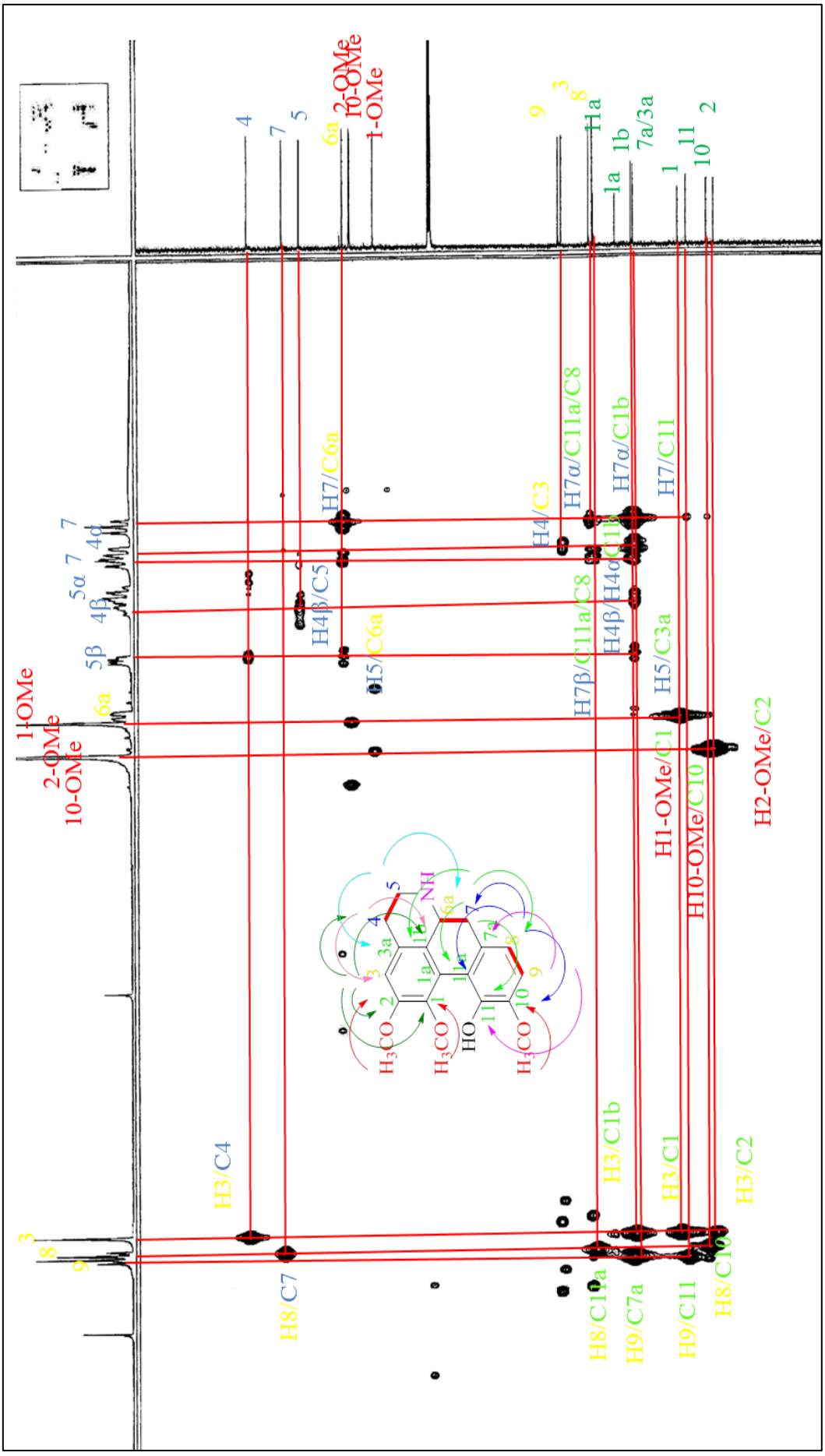
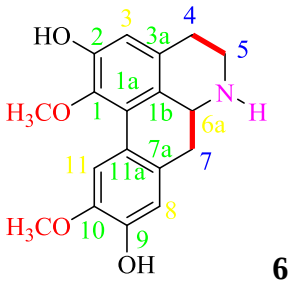


Figure 4.135: HMBC spectrum of norisocorydine (**65**).

4.5.10 Laurolitsine (6)



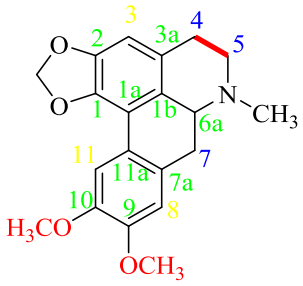
6

Alkaloid **6** was isolated as a brownish amorphous solid. $[\alpha]_D^{25}$ = + 6.77 (c=0.167, MeOH). The UV, IR, MS, 1D-NMR (^{1}H NMR, ^{1}H-NOE-Differential NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) data spectra features were similar to those of laurolitsine (**6**) or norboldine that isolated from dichloromethane extract of bark of *Phoebe grandis* and leaves of *Phoebe tavoyana*. It was described earlier in section **4.3.6** (refer page 203-213).

4.6 Compounds isolated from the fruits of *Actinodaphne sesquipedalis* (HIR 011)

An investigation of fruits from *Actinodaphne sesquipedalis* (HIR 011) gave three known alkaloids and two sterol compounds. The isolation of phytochemicals from the hexane extract of fruits of *Actinodaphne sesquipedalis* afforded four known compounds which consisted largely of dicentrine (**52**); liriodenine (**19**), dicentrinone (**58**) and β-sitosterol (**59**). While from the MeOH extract of the fruit of *Actinodaphne sesquipedalis* (HIR 011) species yielding two sterol compound and two known aporphine alkaloids; namely dicentrine (**52**), β-sitosterol (**59**), dicentrinone (**58**) and stigmasterol (**60**).

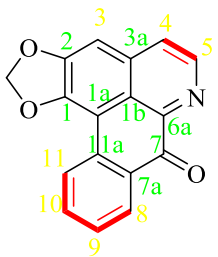
4.6.1 Dicentrine (52)



52

This major alkaloid **52** also isolated from hexane extract of fruits of *Actinodaphne sesquipedalis* crystallized as a colourless prism with the melting point 167-169°C. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC and HSQC) spectral data were identical to dicentrine (**52**) that previously isolated from hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*. It was described earlier in section **4.5.2** (refer page 262-272).

4.6.2 Liriodenine (19)



19

Alkaloid **19** was obtained as fine yellow needles from hexane extract with the m.p. 280-282°C. The LCMS-QTOF mass spectrum (Figure 4.136) showed a molecular ion peak at m/z = 276.0653 $[M+H]^{+}$, consistent with the molecular formula of $C_{17}H_9NO_3$ (calcd for $C_{17}H_{10}NO_3$, 276.0582). An oxoaporphines nature was deduced for this minor alkaloid, based on its intense yellow colour, strongly fluorescent chloroform solution and deep red colouration is produced in acid medium. This was supported by the UV

and IR spectra data (Figure 4.137). The former exhibited absorption bands at 256, 281 and 334 nm. The latter showed a very significant peak at 1636 cm^{-1}. This peak was due to the stretching of a highly conjugated carbonyl group. In addition, the characteristic of a methylenedioxy group were also observed at 1055 and 952 cm^{-1}. A peak at 860 cm^{-1} was present as a result of the C-H out of plane deformation of a single isolated aromatic proton, which is attributed to H-3 (Katsui *et al.*, 1966; Bick & Douglas, 1965).

An integration of the ^{1}H NMR spectrum (Figure 4.138) indicated the presence of 7 aromatic protons with the characteristic of an AB *dd* typical of H-4 and H-5 at δ 7.72 and δ 8.85, respectively, with a coupling constant of 5.2 Hz. An aromatic singlet was observed at δ 7.13 attributed to H-3. A methylenedioxy group attached to C-1 and C-2 gave a singlet of two protons at δ 6.34. Two of one proton *doublet of doublet* (*dd)* at δ 8.58 (*J*=8.0, *J'*=1.5 Hz) and δ 8.54 (*J*=8.0, *J'*=0.7 Hz) were observed assigned to H-11 and H-8 on ring D respectively. H-11 showed the highest chemical shift compared to the other three aromatic protons due to the deshielding effect of the facing ring A and hydrogen bonding with the methylenedioxy group. In addition, two sets of one proton *dt* at δ 7.69 (*J*=8.0, *J'*=1.5 Hz) and δ 7.54 (*J*=8.0, *J'*=0.7 Hz) were observed, which were attributed to H-10 and H-9.

The assignment of alkaloid **19** was further verified by ^{13}C NMR spectrum (Figure 4.139) which exhibited seventeen carbon resonances. Signals at δ 151.7 and δ 147.9 were consistent with two oxygenated aromatic quaternary carbon attached to methylenedioxy group at positioned C-2 and C-1. The presence of the carbonyl group was also evident from the downfield signal at δ 182.42. The ^{13}C NMR and DEPT experiments (Figure 4.139) further confirmed the presence of seven methines, nine

quaternary carbons and methylenedioxy group in the structure. Other carbon signals were assigned using the HSQC spectrum (Figure 4.141).

In the COSY spectra (Figure 4.140) supported the neighbouring proton-proton correlations as shown in Figure 4.140, while the HMBC spectrum was used to assign ^{1}H-^{13}C long range correlations (Figure 4.142). The full assignments for the protons and carbons signals were tabulated in Table 4.20. The compound was identified as liriodenine (**19**) based on these spectral data and comparison with literature values (Hsieh *et al*., 2004; Guo *et al*., 2011). The occurance of this compound was reported as a marker for the family of Annonaceae but, not for Lauraceae family. Interestingly, liriodenine (**19**), an oxoaporphine alkaloid, is reported for the first time from the genus of *A. sesquipedalis* (Lauraceae).

Table 4.20: ^{1}H-NMR (600 MHz),^{13}C-NMR (150 MHz) and 2D (HMBC and HSQC) NMR data of liriodenine (**19**) and the literature data.

	19 in $CDCl_3$				* in $CDCl_3$	
H/C	δ_H (ppm, *J* in Hz)	δ_C (ppm)	HMBC (^2J,^3J)	HSQC (^1J)	δ_H (500MHz) (ppm, *J* in Hz)	δ_C (125MHz) (ppm)
1	-	147.9	-	-	-	147.9
1a	-	108.1	-	-	-	108.4
1b	-	123.2	-	-	-	123.3
2	-	151.7	-	-	-	151.8
3	7.13 (1H, *s*)	103.2	C_4,C_1,C_2	H3	7.19 (1H, *s*)	103.3
3a	-	145.4	-	-	-	145.5
4	7.72 (1H, *d*, *J*=5.2)	124.2	C_3, C_{1b}	H4	7.77 (1H, *d*, *J*=5.1)	124.2
5	8.85 (1H, *d*, *J*=5.2)	144.9	C_4, C_{6a}	H5	8.89 (1H, *d*, *J*=5.1)	145.0
6a	-	135.7	-	-	-	135.8
7	-	182.4	-	-	-	182.4
7a	-	131.3	-	-	-	131.4
8	8.54 (1H, *dd*, *J*=8.0, 0.7)	128.8	$C_{7,}$ C_{10}	H8	8.58 (1H, *d*, *J*=7.8)	128.6
9	7.54 (1H, *dt*, *J*=8.0, 0.7)	128.5	C_{11}, C_{7a}	H9	7.57 (1H, *t*, *J*=7.8)	128.9
10	7.69 (1H, *dt*, *J*=8.0, 1.5)	133.9	C_8, C_{11a}	H10	7.76 (1H, *t*, *J*=8.1)	133.9
11	8.58 (1H, *dd*, *J*=8.0, 1.5)	127.3	C_{1a},C_9	H11	8.65 (1H, *d*, *J*=8.1)	127.4
11a	-	132.9	-	-	-	132.9
OCH_2O	6.34 (2H, s)	102.5	C_1, C_2	$2H_{OCH2O}$	6.37 (2H, s)	102.4

δ_H = chemical shift values in ^{1}H-NMR spectrum; δ_C = chemical shift values in ^{13}C-NMR spectrum.

* (Guo *et al*., 2011).

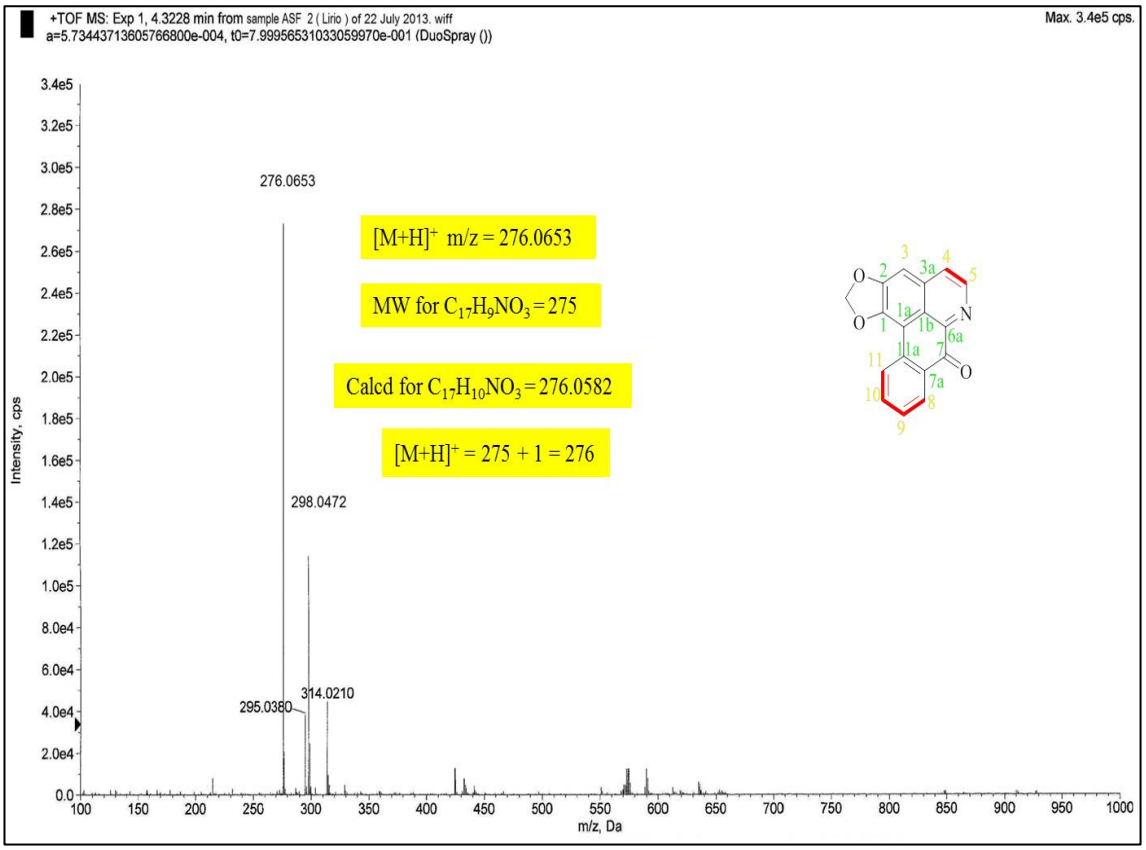


Figure 4.136: LCMS-QTOF spectrum of liriodenine (**19**)

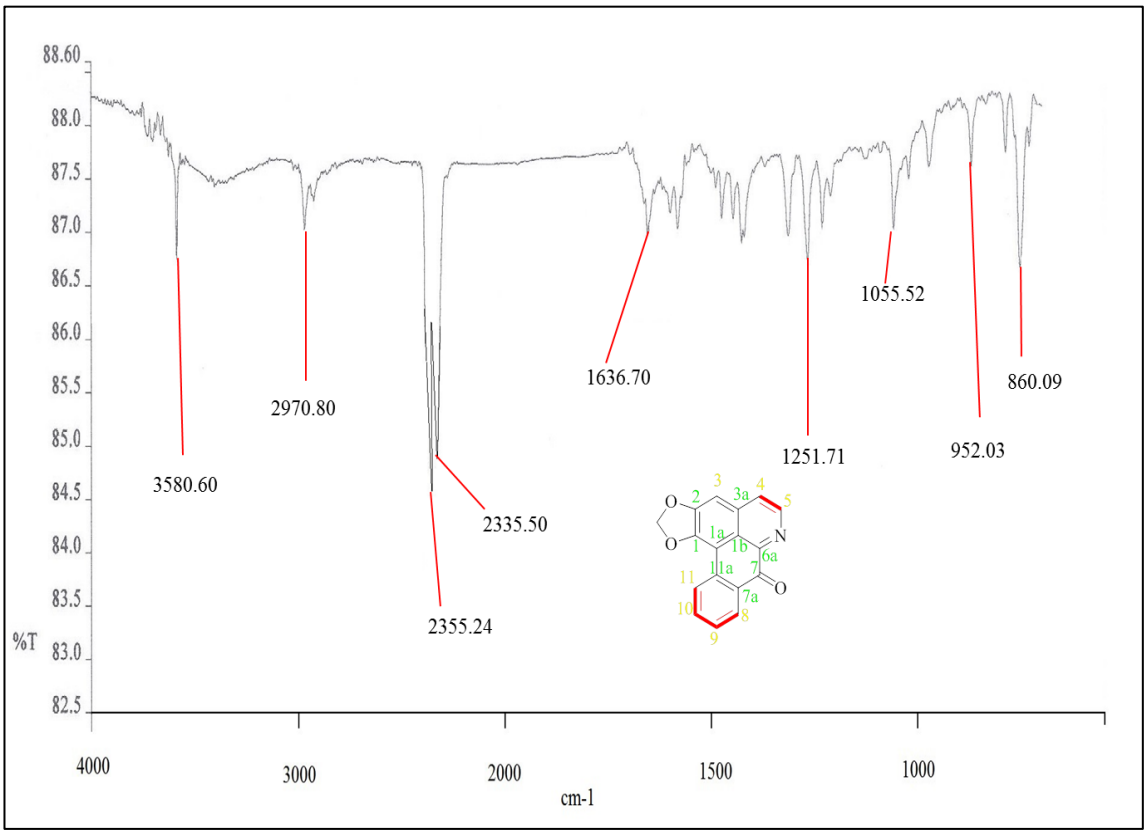


Figure 4.137: IR spectrum of liriodenine (**19**)

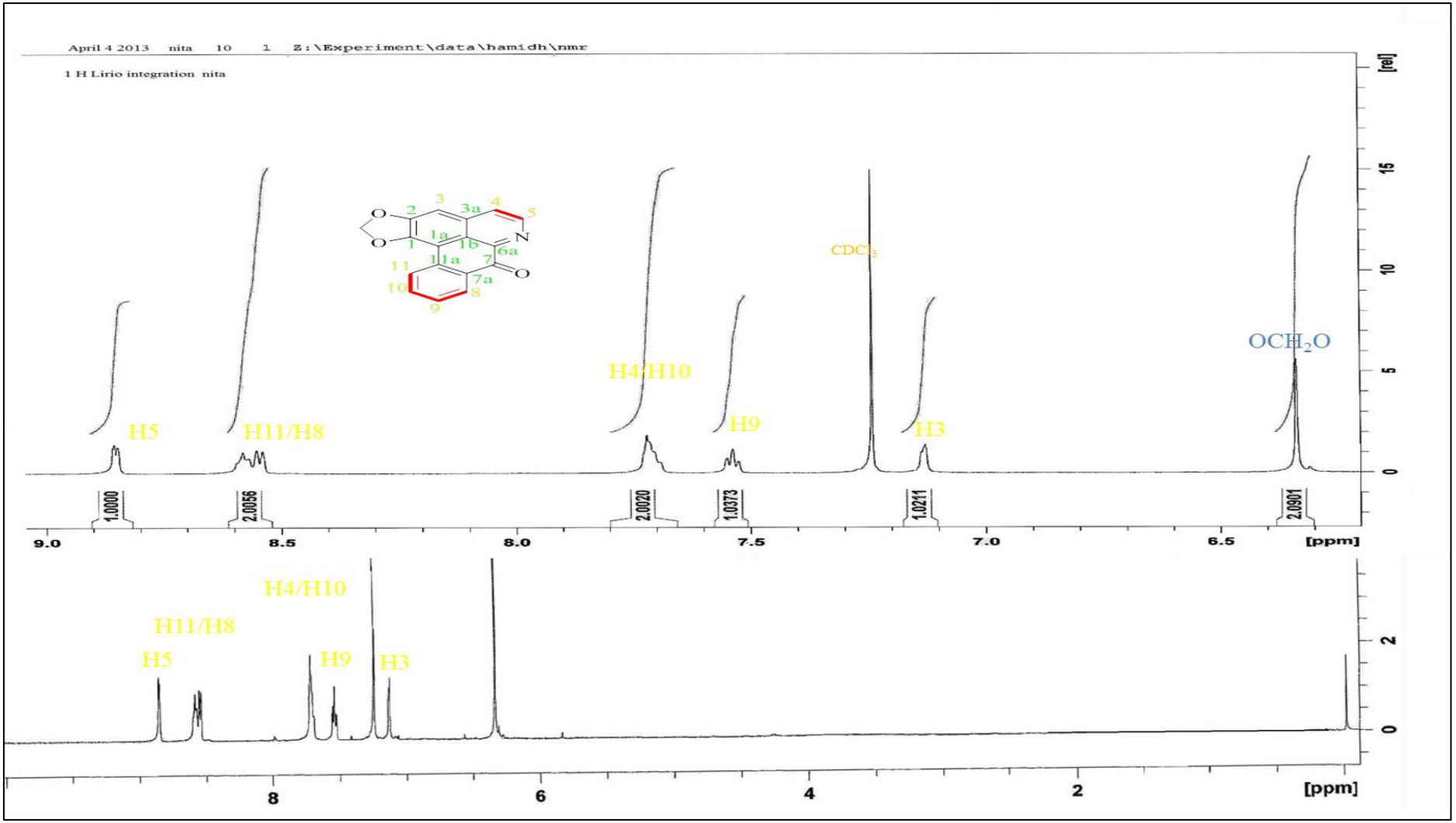


Figure 4.138: [1]H- (integration) NMR spectrum of liriodenine (**19**)

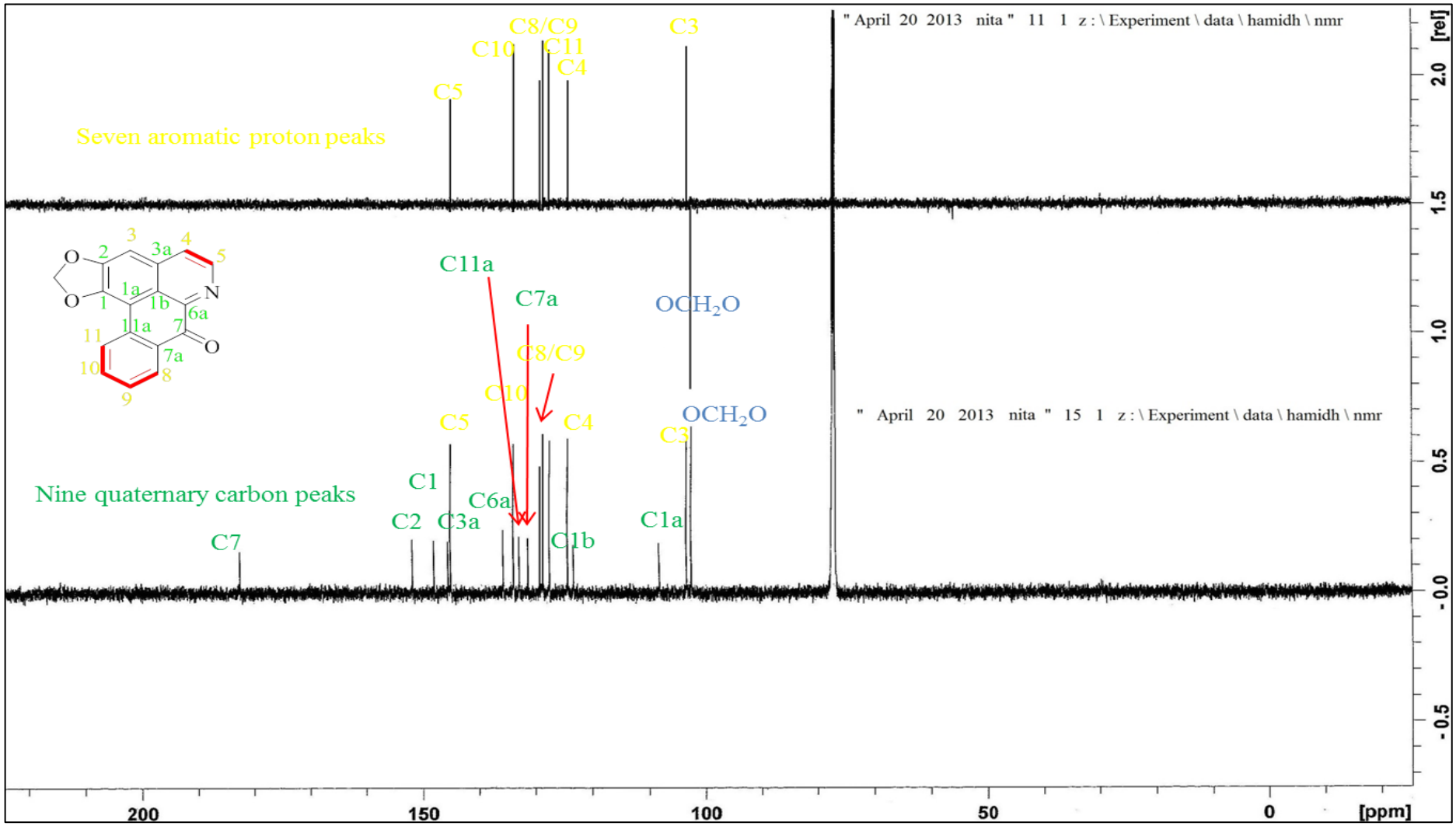


Figure 4.139: ^{13}C and DEPT 135- NMR spectrum of liriodenine (**19**)

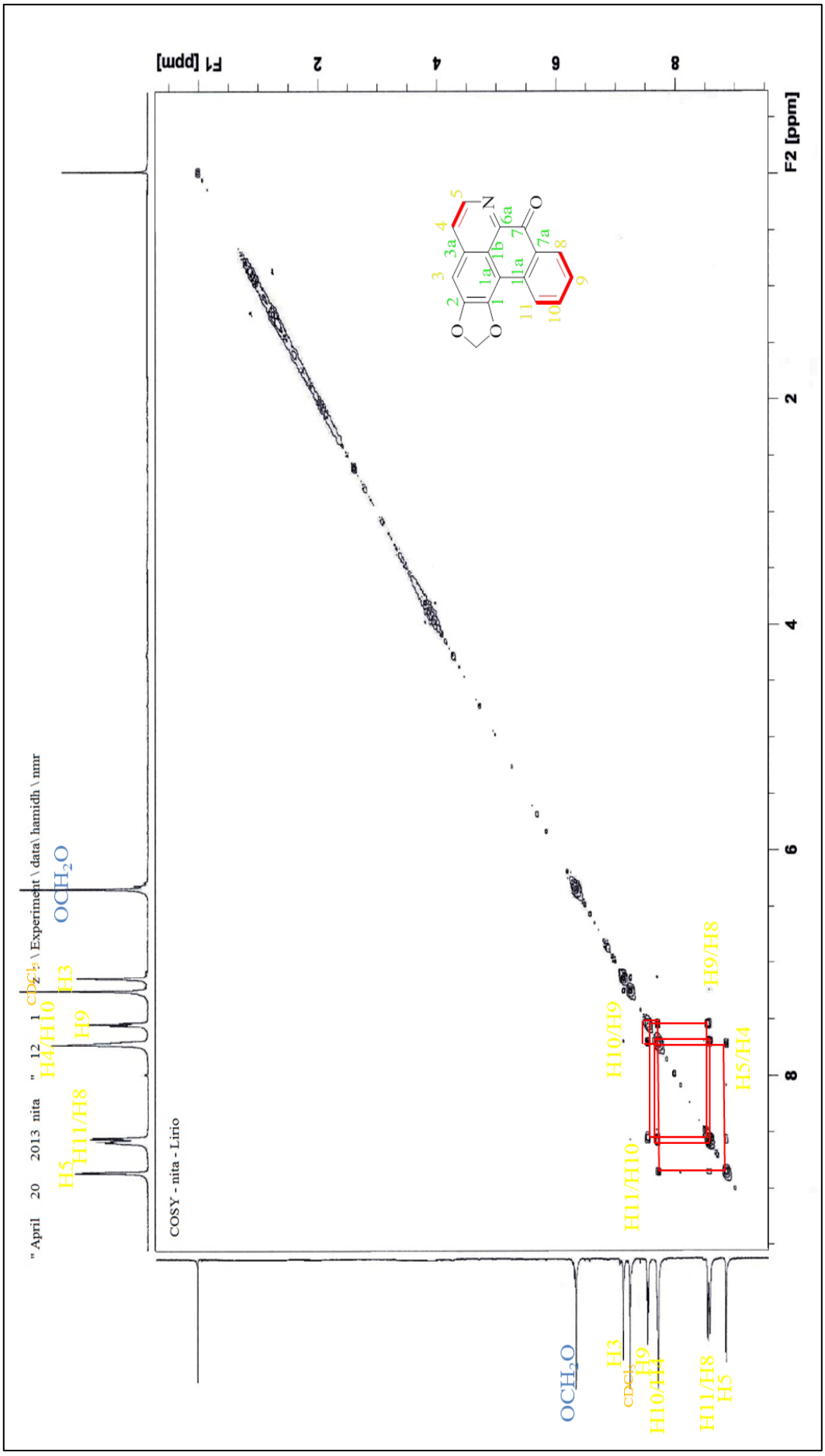


Figure 4.140: ^{1}H- ^{1}H COSY- NMR spectrum of liriodenine (**19**)

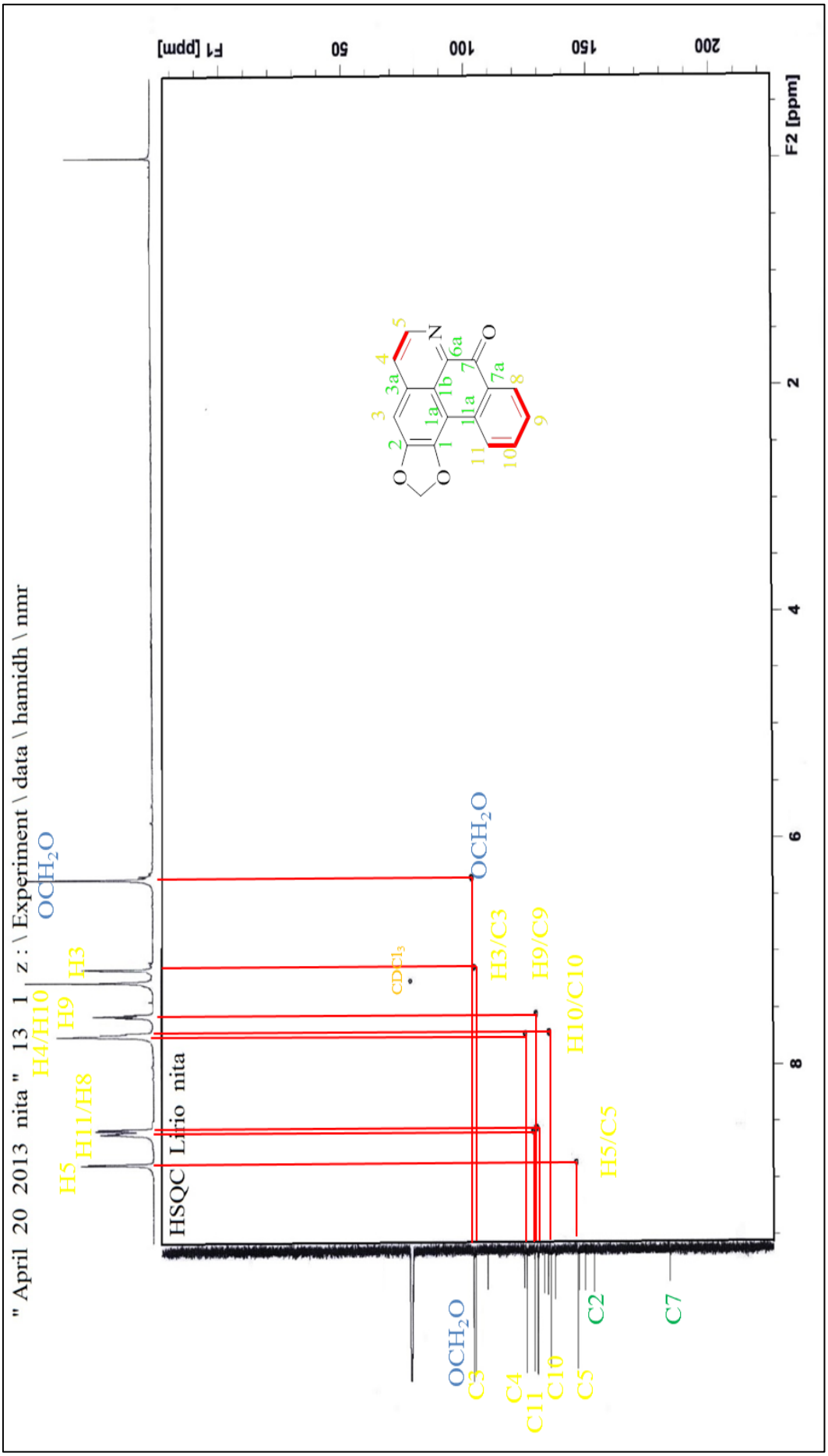


Figure 4.141: ^{1}H- ^{13}C HSQC- NMR spectrum of liriodenine (**19**)

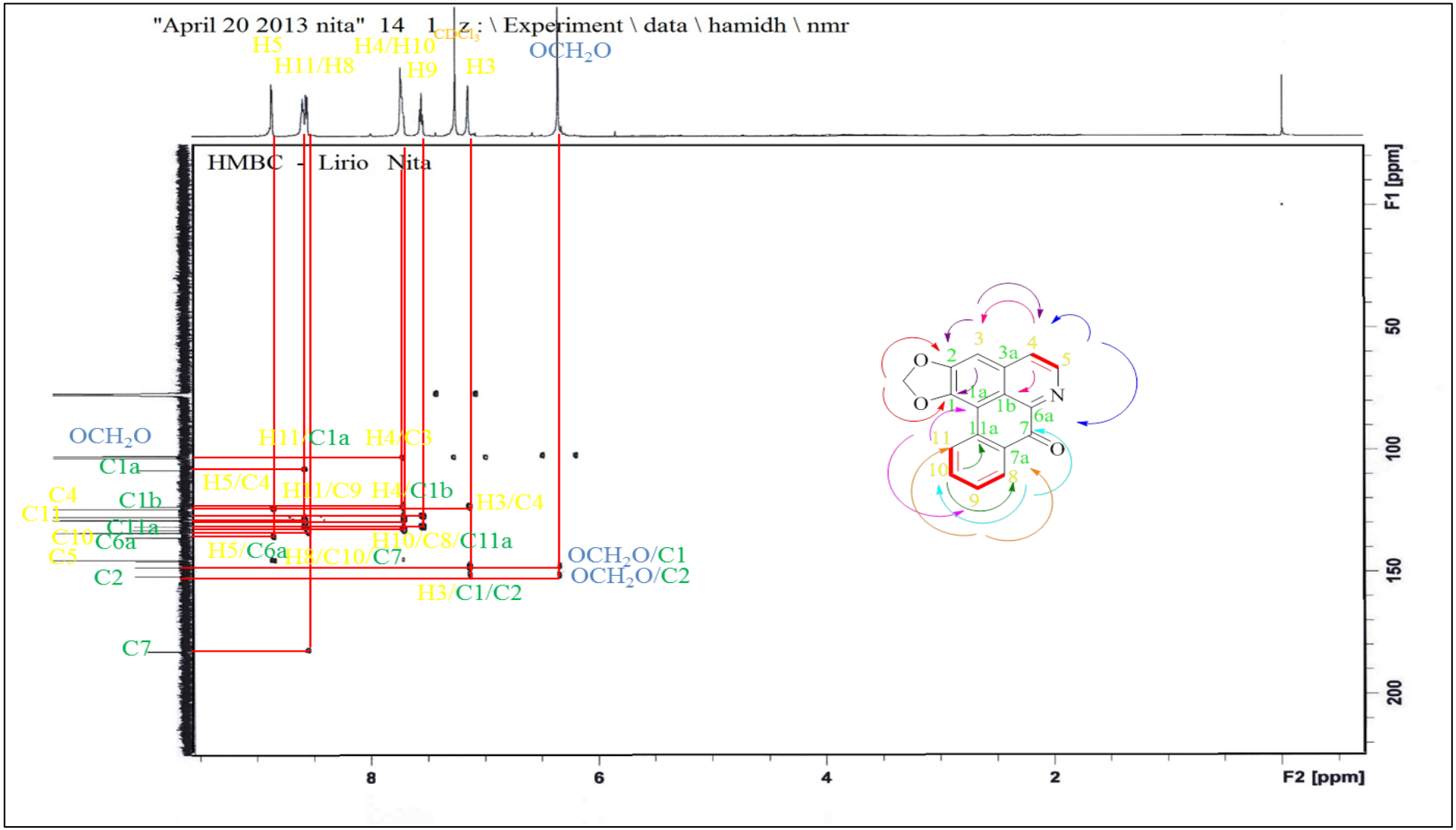
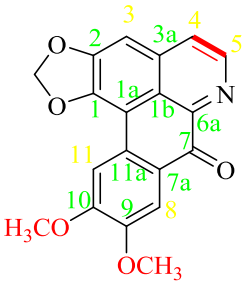


Figure 4.142: ^{1}H-^{13}C HMBC- NMR spectrum of liriodenine (**19**)

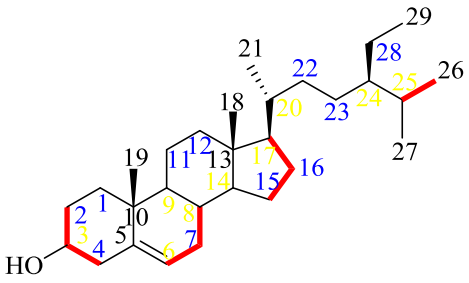
4.6.3 Dicentrinone (58)



58

Compound **58** was obtained as fine yellow needles. The UV, IR, MS, 1D-NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D-NMR (COSY, HMBC and HSQC) data spectra features were identical to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis* and methanol extract of leaves of *Actinodaphne sesquipedalis*. In fact dicentrinone (**58**) has been discussed previously in section **4.2.5** (refer page 147 - 155).

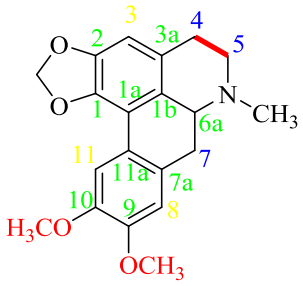
4.6.4 β-sitosterol (59)



59

Compound **59** with $[\alpha]^{25}_{D}$ = -36.6˚ (c= 0.15, $CHCl_3$) was isolated from hexane extract of fruits of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) spectral data were identical to β-sitosterol (**59**) isolated from hexane extract of bark of *Phoebe grandis*; and hexane and methanol extract of leaves of *Actinodaphne sesquipedalis* and previously has been discussed in section **4.3.1** (refer page 156 - 168).

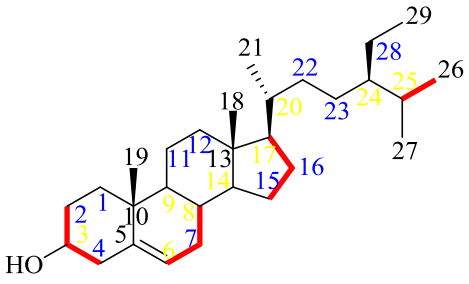
4.6.5 Dicentrine (52)



52

This major alkaloid **52** also isolated from the methanol extract of fruits of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC and HSQC) spectral data were identical to dicentrine (**52**) that previously isolated from hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*; and hexane extract of fruits of *Actinodaphne sesquipedalis* It was described earlier in section **4.5.2** (refer page 262 - 272).

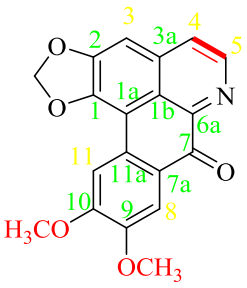
4.6.6 β-sitosterol (59)



59

Compound **59** with $[\alpha]^{25}_{D}$ = -36.6˚ (c= 0.15, $CHCl_3$) was isolated from methanol extract of fruits of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) and 2D-NMR (COSY, HMBC, HSQC and NOESY) spectral data were identical to β-sitosterol (**59**) isolated from hexane extract of bark of *Phoebe grandis*; hexane and methanol extract of leaves of *Actinodaphne sesquipedalis*; and hexane extract of fruits of *Actinodaphne sesquipedalis*. Previously it has been discussed in section **4.3.1** (refer page 156 - 168).

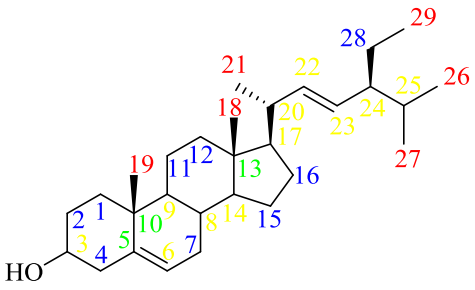
4.6.7 Dicentrinone (58)



58

Compound **58** was obtained as fine yellow needles. It was isolated from methanol extract of fruits of *Actinodaphne sesquipedalis*. The UV, IR, MS, 1D-NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D-NMR (COSY, HMBC and HSQC) data spectra features were identical to dicentrinone (**58**) isolated from dichloromethane extract of leaves of *Phoebe grandis*; methanol extract of leaves of *Actinodaphne sesquipedalis*; hexane and methanol extract of fruits of *Actinodaphne sesquipedalis*. Dicentrinone (**58**) has been discussed previously in section **4.2.5** (refer page 147 - 155).

4.6.8 Stigmasterol (60)



60

Compound **60** was isolated from the methanol extract of the fruits of *Actinodaphne sesquipedalis.* The UV, IR, MS, 1D-NMR (^{1}H NMR and ^{13}C NMR) spectral data were identical to stigmasterol (**60**) that isolated from hexane extract of bark of *Phoebe*

grandis and hexane extract of leaves of *Actinodaphne sesquipedalis*. Stigmasterol (**60**) has been discussed previously in section **4.3.2** (refer page 169 - 175).

4.7 Biological activity of crude extracts

Several bioassays, including cytotoxicity, antioxidant activity and antibacterial activity were performed to evaluate potential bioactivity of *Phoebe grandis*, *Phoebe tavoyana* and *Actinodaphne sesquipedalis.*

4.7.1 Cytotoxicity Activity

Twenty extracts of the *Phoebe grandis*, *Phoebe tavoyana* and *Actinodaphne sesquipedalis* were analysed for their cytotoxic activity against three cancer cell lines, MCF-7 (human estrogen receptor (ER+) positive breast cancer), Caov-3 (Human Ovarian cancer cell line) and HepG2 (Human Liver cancer). The cytotoxic activities were carried out by using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) method. The evaluation of cytotoxic activity is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product which can be measured spectrophotometrically. The mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan and the amount of formazan produced is directly proportional to the number of viable cells (Lee *et al.*, 2004). The IC_{50} value (the drug concentration causing 50% inhibition of the tumour cell) was used as a parameter for cytotoxicity, after 24 hrs treatment with different concentration of extracts. The IC_{50} values obtained from the plotted graphs (see **Appendix A**), showed the concentration or the cytotoxic

dose that was able to reduce the cell population to 50%. The reference drugs used in this assay were tamoxifen, paclitaxel and doxorubicin.

The IC_{50} values of the crude extracts against MCF-7, Caov-3 and HepG2 cells are presented in Table 4.21. The displayed cytotoxic activity of the extracts was different and varies. According to National Cancer Institute (NCI) guidelines, plant extracts with IC_{50} less than 20 µg/mL were considered active (Geran, 1972). Most of the results showed the percentage of cell viability increased as the concentration of the extracts decrease and the graphs were plotted to get the IC_{50} values. The results imply that the cells became less sensitive towards lower concentration of extracts. However, the moderate results from Table 4.21 showed that the dichloromethane extract from leaves of *A. sesquipedalis*, (HIR 011) was less sensitive towards MCF7 cell line with the IC_{50} value 30.00 µg/mL, but did not show any activity against Caov-3 and HepG2 cell lines. Other extracts displayed moderate activity against MCF7 cells were the dichloromethane extract (acid-base) from bark of *P. tavoyana* (KL 5225), dichloromethane extract (acid-base) from bark of *P. grandis* (KL 4994) and chloroform extract (acid-base) from bark of *P. tavoyana* (KL 5225) with IC_{50} values 40.00, 45.00 and 48.00 µg/mL, respectively.

The dichloromethane extract (acid-base) from bark of *P. tavoyana* (KL 5225) also showed cytotoxic effect towards Caov-3 with the IC_{50} value 35.00 µg/mL, while the dichloromethane extract (acid-base) from bark of *P. grandis* (KL 4994) gave the IC_{50} value of 45.00 µg/mL. These results considered as moderately active against Caov-3. The other tested extracts from *P. grandis*, *P. tavoyana* and *A. sesquipedalis* were either inactive or require higher concentration to inhibit the viability of Caov-3 cells in culture, as the IC_{50} values were > 100.00 µg/mL.

However, most of the plants do not render toxic effects on the HepG2 (Human Liver cancer) cells with IC_{50} values more than 100.00 µg/mL. The activities as summarised in the Table 4.21, showed that all of the crude extracts did not show any activity, very weak activity or the IC_{50} are > 100.00 µg/mL against HepG2, except dichloromethane extract from fruits of *A. sesquipedalis* (HIR 011) exhibited weak activity with IC_{50} value of 80.00 µg/mL. It could be due to the chemical constituents from the crude extracts do not contained compound with the substituent that contributes to biological activity towards HepG2, or may be constituents with structure-active relationships (SAR) exist with small quantity compared with other constituents.

It was reported that (+)-N-(2-hydroxypropyl)lindcarpine from *Actinodaphne pruinosa* Nees exhibited cytotoxic activity against P-388 murine leukemia cells with an IC_{50} value of 3.9 µg/ml (Omar *et al*., 2013). This study supported the previous results and need more detail investigations on *Actinodaphne sesquipedalis* against cancer cell lines, HepG2 since IC_{50} value of 80.00 µg/mL showed weak activity (see **Appendix A**). Fruits of *A. sesquipedalis*, (HIR 011) may contain promising therapeutic anticancer.

None of the tested cancer cell lines, showed activity against hexane extracts from *P. grandis, P. tavoyana* and *A. sesquipedalis*. On the basis of the screening results and literature review on the tested plant, *P. grandis* was selected for further phytochemical investigations and isolated compounds from *P. grandis* leaves were analysed for their cytotoxic activity against cancer cell lines, MCF-7 and HepG2.

Table 4.21: The IC_{50} values of MCF-7, Caov-3 and HepG2 of extracts of selected Lauraceae species using MTT assay (at 24 hrs treatment).

SAMPLES	Type of extract	IC_{50} Values (µg/ml)			
		Code	MCF-7	Caov-3	HepG2
P. grandis (leaves) KL 5440	Hexane	S20	>100	>100	>100
	CH_2Cl_2	S18	>100	>100	>100
	CH_2Cl_2 (acid-base)	S17	>100	>100	>100
	MeOH	S19	>100	>100	>100
P. grandis (bark) KL 4994	CH_2Cl_2 (acid-base)	S4	45.00	45.00	>100
P. tavoyana (leaves) KL 5225	Hexane	S15	>100	>100	>100
	CH_2Cl_2	S16	>100	>100	>100
	MeOH		>100	>100	>100
P. tavoyana (bark) KL 5225	Hexane	S9	>100	>100	>100
	CH_2Cl_2	S8	>100	>100	>100
	CH_2Cl_2 (acid-base)	S11	40.00	35.00	>100
	$CHCl_3$ (acid-base)	S14	48.00	>100	>100
A. sesquipedalis (leaves) HIR 011	Hexane	S10	>100	>100	>100
	CH_2Cl_2	S6	30.00	>100	>100
	MeOH	S5	>100	>100	>100
A. sesquipedalis (bark) HIR 011	MeOH	S13	>100	>100	>100
	MeOH (acid-base)	S3	>100	>100	>100
A. sesquipedalis (fruits) HIR 011	Hexane	S7	>100	>100	>100
	CH_2Cl_2	S1	>100	>100	80.00
	Ethyl acetate	S2	>100	>100	>100
	MeOH	S12	>100	>100	>100
Doxorubicin			-	-	1.06
Paclitaxel			-	<0.79±0.1	-
Tamoxifen			1.01±1.21	-	-

MCF7: human estrogen receptor (ER+) positive breast cancer.
Caov-3: Human Ovarian cancer cell line.
HepG2: Human Liver cancer.

4.7.2 DPPH free radical scavenging activity

In this study, the antioxidant properties of extracts were evaluated spectrophotometrically using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. The antioxidant capacities of the extracts were assayed *in vitro* for their ability to scavenge the DPPH free radical, through the donation of proton forming the reduced DPPH. The free radical scavenging activity is usually expressed as percentage DPPH inhibition but also by the concentration (µg/mL) of extracts required to inhibit the formation of DPPH radicals by 50 % as IC_{50}. The IC_{50} value was calculated from the graph plotted inhibition percentage against extract concentration. The control intensities of the extracts were assumed to be 100 % and the percentage inhibition calculated. The positive control used in this assay was ascorbic acid.

The scavenging activities and the IC_{50} values of the extracts are displayed in Table 4.22. However, none of the crude extracts of *P. grandis*, *P. tavoyana* and *A. sesquipedalis* exhibited notable free radical scavenging activity; except (acid-base) dichloromethane extract from *P. grandis* (bark) was observed with the IC_{50} value of 486.8 µg/mL (not active). The colour changes from purple to yellowish after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The weak activity observed in the DPPH radical scavenging assay may be as a result of the low level of the potency of the plant extracts in donating hydrogen proton to the lone pair electron of the radicals. The electrons become paired off and solution loses colour stochiometrically depending on the number of electrons taken up. The inhibition value was more at a higher concentration in all the solvent extracts, it could be suggested that the plant extracts still contain small amount of compounds capable of donating protons to the free radicals. DPPH was used to determine the proton radical scavenging action of extracts

of the plants, because it possesses a proton free radical and shows a characteristic absorbance at 517 nm.

The probable reason for the lower DPPH values of the extracts samples could be due to the presence of compounds not reactive towards DPPH. Antioxidant compounds such as polyphenol may be more efficient as reducing agents for ferric iron but some may not scavenge DPPH free radicals as efficiently due to steric hindrance. In this study, results imply that the radical scavenging activities of the extracts from *Phoebe* and *Actinodaphne* species may be attributed to the phytochemicals composition in proton or electron donating abilities. As mention earlier, *Actinodaphne* species are rich in flavonoids and *Phoebe* species have been used in the traditional medicine for wounds and sores (Omar *et al.*, 2013). These extracts are believed to most likely contribute to the antioxidant activity besides other extracts.

Table 4.22: The IC_{50} values and percentages inhibition of DPPH (free radical) treated with different concentration of extract of *Phoebe grandis*, *Phoebe tavoyana* and *Actinodaphne sesquipedalis* measured after 20 minutes.

Concentration (µg/ml)		Inhibition (%)						
Plant species	Extracts	500	250	125	62.5	31.3	15.6	IC_{50} (µg/ml)
P. grandis (leaves) 5440	Hexane	6.71	17.85	10.76	5.94	0.15	4.51	>500
	CH_2Cl_2	4.11	12.80	36.45	0.00	0.00	0.00	>500
	CH_2Cl_2 (acid-base)	20.13	14.39	13.56	1.62	5.44	6.67	>500
	MeOH	14.26	0.00	40.88	0.00	3.68	17.26	>500
P. grandis (bark) 4994	CH_2Cl_2 (acid-base)	51.35	31.46	12.89	0.00	0.00	8.78	486.8
P. tavoyana (leaves) 5225	Hexane	5.48	0.00	0.00	0.00	0.00	0.00	>500
	CH_2Cl_2	7.23	4.04	0.00	0.00	0.00	0.00	>500
P. tavoyana (bark) 5225	Hexane	7.58	19.27	21.53	21.27	4.06	10.62	>500
	CH_2Cl_2	0.00	8.93	5.04	14.20	0.00	0.00	>500
	CH_2Cl_2 (acid-base)	18.42	23.24	16.21	12.51	7.04	1.81	>500
A.sesquipedalis (leaves) 011	Hexane	22.19	9.25	0.00	1.37	0.44	1.43	>500
	CH_2Cl_2	48.19	13.21	15.32	4.66	3.89	0.88	>500
	MeOH	45.21	52.88	41.85	25.44	13.16	2.09	>500
A.sesquipedalis (bark) 011	MeOH	34.36	32.65	15.08	6.18	6.83	3.00	>500
	MeOH (acid-base)	23.68	30.00	14.08	13.18	3.23	4.43	>500
A.sesquipedalis (fruits) 011	Hexane	0.00	2.01	0.00	0.00	0.00	0.00	>500
	CH_2Cl_2	0.00	0.00	0.00	0.00	0.00	0.00	>500
	Ethyl acetate	34.15	0.00	0.00	0.00	0.00	0.00	>500
	MeOH	35.13	25.77	13.56	2.97	0.00	0.00	>500
Standard Ascorbic acid		85.39	86.55	86.67	86.86	51.18	26.66	30.5

(Note: Data presented are the mean of triplicates of percentage of inhibition).

4.7.3 FRAP assay (ferric reducing ability of plasma)

The reductive capabilities of the extracts of leaves and bark of *Phoebe grandis* and *Phoebe tavoyana*; leaves, bark and fruits of *Actinodaphne sesquipedalis* were compared with ascorbic acid. The reducing capacity Fe^{3+} to Fe^{2+} of the extracts may serve as a significant indicator of its potential antioxidant activity. The reducing power of the extract samples increased with increasing amount of sample (Baskar *et al*., 2007). The antioxidant activity has been reported to be concomitant with the development of reducing power.

The FRAP values of the studied fractions were calculated and results are shown in Table 4.23. Graph of the FRAP values of extracts measured after 4 minutes incubation (see **Appendix B**). Among all the fractions, the dichloromethane (acid-base) fraction of *Phoebe tavoyana* (bark) showed highest FRAP value (3557.94 $\pm$ 0.07). The dichloromethane (acid-base) fraction of *Phoebe grandis* leaves and bark; ethyl acetate fraction from *Actinodaphne sesquipedalis* (fruit) showed FRAP values of 1594.89 $\pm$ 0.04, 1585.61 $\pm$ 0.00 and 1432.17 $\pm$ 0.01, respectively, which are almost closed to standard ascorbic acid value 1145.61 $\pm$ 0.06. From the FRAP values, n-hexane fraction was found to be poor activity compared to other extracts. High FRAP values obtained for more polar extracts may be ascribed partially to the presence of phenolic and flavonoid contents.

In this study, hexane extract of *Phoebe tavoyana* (bark) displayed the lowest reducing power and alkaloid extract or dichloromethane extract (acid-base) of *Phoebe tavoyana* (bark) had the highest ferric ions reducing activity. To date, the potent antioxidant properties of *Phoebe tavoyana* has not been reported.

Table 4.23: The FRAP values of extracts of *Phoebe grandis*, *Phoebe tavoyana* and *Actinodaphne sesquipedalis* measured after 4 minutes incubation.

Plant species	Extracts	Code	FRAP value (mM Fe(II)/mg extract)	SEM	FRAP value ± SEM
P. grandis (leaves)	Hexane	PGL-A	187.8333	0.0029	187.83 ± 0.00
	CH_2Cl_2	PGL-B	662.0556	0.0341	662.06 ± 0.03
	CH_2Cl_2 (acid-base)	PGL-B(ACID)	1594.8889	0.0353	1594.89 ± 0.04
	MeOH	PGL-D	64.7778	0.0026	64.78 ± 0.00
P. grandis (bark)	CH_2Cl_2 (acid-base)	PGB-B (SAME PGB)	1585.6111	0.0042	1585.61 ± 0.00
P. tavoyana (leaves)	Hexane	PTL-A	74.3889	0.0079	74.39 ± 0.01
	CH_2Cl_2	PTL-B	365.5556	0.0127	365.56 ± 0.01
P. tavoyana (bark)	Hexane	PTB-A	48.1111	0.0017	48.11 ± 0.00
	CH_2Cl_2	PTB-B	3047.3889	0.1042	3047.39 ± 0.10
	CH_2Cl_2 (acid-base)	PTB-B (ACID)	3557.9444	0.0691	3557.94 ± 0.07
A. sesquipedalis (leaves)	Hexane	HIRL-A	355.7778	0.0137	355.78 ± 0.01
	CH_2Cl_2	HIRL-B	892.1667	0.0211	892.17 ± 0.02
	MeOH	HIRL-D	1821.8889	0.1635	1821.89 ± 0.16
A. sesquipedalis (bark)	MeOH	HIRB-D	2810.2778	0.0825	2810.28 ± 0.08
	MeOH (acid-base)	HIRB-D(ACID)	3128.7778	0.0569	3128.78 ± 0.06
A. sesquipedalis (fruits)	Hexane	HIRF-A	57.6667	0.0029	57.67 ± 0.00
	CH_2Cl_2	HIRF-B	570.8889	0.0168	570.89 ± 0.02
	Ethyl acetate	HIRF-C	1432.1667	0.0064	1432.17 ± 0.01
	MeOH	HIRF-D	1756.1111	0.0823	1756.11 ± 0.08
Standard Ascorbic acid			1145.6111	0.0557	1145.61 ± 0.06

(Note: Data presented are the mean of triplicates of extracts sample – three different concentrations).

4.7.4 Antibacterial activity assay

Extracts from 3 plants belonging to Lauraceae family were investigated for their antibacterial activity against six common bacteria. They were *Bacillus subtilis* (B145-IMR culture), *Staphylococcus aureus* (S1434-IMR culture), *Staphylococcus epidermidis* (a clinically isolated strain-UMMC) and methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC BAA-1720) from (gram-positive) type; while, *Escherichia coli* (ATCC 25922) and *Salmonella typhi* (clinically isolated strain-UMMC) were (gram-negative) type.

The results of antibacterial activities of the extracts of these plants are presented in Table 4.24. This antibacterial activity was quantitatively determined by the presence or absence of inhibition zone around the discs containing extract (clear zone of growth inhibition). An inhibition zone > 15 mm was considered as a high antibacterial activity. The results exhibited that *B. subtilis* (gram-positive) was the most resistant strains among 6 tested bacterial species and followed by *E. coli* (gram-negative) and *S. epidermidis* (gram-positive). All the extracts did not show considerable inhibitory activity on *B. subtilis* (gram-positive) however, they displayed significant antibacterial effect against *S. aureus* S1434 (gram-positive) and MRSA (gram-positive). Only hexane extracts from *A. sesquipedalis* (fruit), *P. tavoyana* (leaves) and *P. grandis* (leaves) displayed antibacterial activity against *S. typhi* (gram negative bacteria); and dichloromethane extracts from *P. tavoyana* (leaves) and *P.grandis* (bark) exhibited potential activity against *E. coli* (gram negative) bacteria.

In this study, extract of *P. grandis* bark (crude alkaloid) had most significant antibacterial activity against both gram positive and gram negative organism except for

B. subtilis (gram-positive) and the activity was comparable to the positive controls, gentamycin and streptomycin sulphate. The activity of *P. grandis* leaves (crude alkaloid) was categorised as greatest activity against bacteria gram-positive, *S. aureus* S1434 (16.67±0.01), *S. epidermidis* (15.67±0.01), and methicillin resistant *Staphylococcus aureus* (MRSA) (14.00±0.01); but no appreciable zone of inhibition was observed with bacteria gram negative, *E. coli* and *S. typhi*. The present study indicates that *Phoebe* species possessed strong antibacterial activity. Therefore, further evaluation of the antibacterial properties of these extracts should be done and focus on compounds that isolated from the *P. grandis* leaves (crude alkaloid) extract. To the best of our knowledge, there is no previous report on the antibacterial activities of the *P. tavoyana* and *A. sesquipedalis*.

The resistance of gram negative bacteria towards plant extracts has been documented previously (Paz *et al*., 1995; Vlietinck *et al.*, 1995; Kudi *et al*., 1999) and is supported by this study. These observations are likely to be the result of the differences in cell wall structure between gram positive and gram negative bacteria, whereby in the gram negative bacteria, outer membrane cell acting as barrier to many environmental substances, including antibiotics (Tortora *et al*., 2001).

Other studies, have also found that some species of *Phoebe* in China, Indonesia, Indochina, Japan, the Philippines and Malay Peninsular to be useful to treat several diseases such as antidiabetic, antibacterial and antifungal activities (Semwal *et al*., 2008). Similar findings have been recorded by another group in respect to ethanolic extract of *P. lanceolata* bark showed effective antibacterial activity against selected gram positive and gram negative bacteria (including *S.aureus, S.mutans, S.epidermidis, E.coli* and *K.pneumonie*) with MIC range 50-100 µg/mL (Semwal *et al*., 2009).

Table 4.24: The diameter of inhibition zone (in mm) of crude extracts from *P. grandis*, *P. tavoyana* and *A. sesquipedalis* against pathogenic microbes.

Plant species	Extracts	Diameter of inhibition zone (in mm)					
		B.subtilis B145	*S.aureus S1434*	*S.epidermidis*	*E.coli ATCC 25922*	*S. typhii*	*MRSA ATCC BAA-1720*
P.grandis leaves, (KL 5440)	Hexane	NI	NI	NI	NI	9.00±0.01	NI
	CH_2Cl_2	NI	NI	NI	NI	NI	NI
	CH_2Cl_2 (acid-base)	NI	16.67±0.01	15.67±0.01	NI	NI	14.00±0.01
	MeOH	NI	NI	NI	7.00±0.01	NI	NI
P.grandis bark, (KL 4994)	CH_2Cl_2 (acid-base)	NI	13.33±0.01	15.00±0.01	14.00±0.01	9.67±0.01	10.00±0.01
P.tavoyana leaves, (KL 5225)	Hexane	NI	7.00±0.01	NI	NI	10.00±0.01	8.33±0.01
	CH_2Cl_2	NI	NI	NI	NI	9.00±0.01	NI
P.tavoyana bark, (KL 5225)	Hexane	NI	7.67±0.01	NI	NI	NI	8.33±0.01
	CH_2Cl_2	NI	7.33±0.01	NI	NI	NI	8.00±0.01
	CH_2Cl_2 (acid-base)	NI	9.00±0.01	NI	NI	NI	7.33±0.01
	$CHCl_3$ (acid-base)	NT	NT	NT	NT	NT	NT
A.sesquipedalis leaves, (HIR 011)	Hexane	NI	NI	NI	NI	NI	NI
	CH_2Cl_2	NI	NI	NI	NI	NI	NI
	MeOH	NI	9.33±0.01	NI	NI	NI	9.00±0.01
A.sesquipedalis bark, (HIR 011)	MeOH	NI	10.00±0.01	NI	NI	NI	9.00±0.01
	MeOH (acid-base)	NI	NI	NI	NI	NI	NI
A.sesquipedalis fruits, (HIR 011)	Hexane	NI	7.00±0.01	NI	NI	7.00±0.01	7.00±0.01
	CH_2Cl_2	NI	8.67±0.01	NI	NI	NI	8.00±0.01
	Ethyl acetate	NI	NI	NI	NI	NI	NI
	MeOH	NI	8.00±0.01	NI	NI	NI	8.00±0.01
Gentamicin*		**30.00± 0.01**	**22.00±0.01**	**24.00±0.01**	**18.00±0.01**	**22.33±0.57**	**23.00±0.01**
Streptomycin*		**NT**	**22.00±0.01**	**23.00±0.01**	**18.00±0.01**	**10.00±0.01**	**17.00±0.01**

Note: The crude extracts to be tested were dissolved in dimethylsulphoxide (DMSO) at concentration of 100 mg/mL. Data presented are the mean of triplicates.
*Positive control.
NI= No inhibition zone.
NT=Not tested.

4.8 Biological activity of isolated compounds

The isolated compounds were evaluated for their cytotoxic effect, antibacterial and antiplasmodial activities. Only four compounds from *P.grandis* leaves, namely lysicamine (**54**), litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) were tested for cytotoxic effect and antibacterial activity. Referring to the previous studies, lysicamine (**54**), litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56**), hexahydromecambrine A (**57**) and dicentrinone (58) were the first time isolated compounds from the leaves of *P. grandis*. While, litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**) were isolated as new naturally occurring compounds from leaves of *P. grandis*.

Antiplasmodial activity was conducted on the isolated compounds from CH_2Cl_2 extract of *P. tavoyana* leaves. The isolated compounds were tested for *in vitro* inhibitory activity against *P. falciparum* 3D7.

Other compounds were not evaluated due to inadequate amount of the compound and not available for screening.

4.8.1 Cytotoxicity activity

A preliminary screening against cancer cells was performed using the Microculture Tetrazolium Salt (MTT) assay, method (Mosmann, 1983) against MCF7 and HepG2 cell lines. The cytotoxicity of compounds lysicamine (**54**), litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) were assayed at various concentrations under continuous exposure for 24 h, are expressed in IC_{50} values

(μg/mL), and summarised in Table 4.25. Doxorubicin was used as a positive control in this study. The results showed compound lysicamine (**54**) and litsericinone (**55**), exhibited cytotoxic activity against both cancer cell lines with IC_{50} values ranging from 14 – 60 μg/mL. Compound 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) exhibited cytotoxic activity against the HepG2 cell line with IC_{50} values of 81 and 20 μg/mL, respectively. Compound 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**), however, were not toxic towards MCF7 cell line with IC_{50} values of more than 100 μg/mL. (see **Appendix C**).

Table 4.25: The IC_{50} values of isolated compounds towards MCF7 and HepG2 cell lines.

Compounds	The IC_{50} (μg/mL) at 24 h	
	MCF7	HepG2
Lysicamine (**54**)	26	27
Litsericinone (**55**)	60	14
8,9,11,12-tetrahydromecambrine (**56**)	> 100	81
Hexahydromecambrine A (**57**)	> 100	20
Doxorubicin*	0.2	1.06

IC_{50}: 50% inhibitory concentration
IC_{50} greater than 100 μg/mL were not active.
*Positive control

Table 4.25 shows an aporphine alkaloid lysicamine (**54**) demonstrated moderate activity against MCF7 and HepG2 cell lines. Meanwhile litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**) showed weak cytotoxicity against MCF7 cell lines. However, litsericinone (**55**) and hexahydromecambrine A (**57**) showed moderate activity against HepG2 cells. It is important to notice that compounds litsericinone (**55**), 8,9,11,12-tetrahydromecambrine

(**56)** and hexahydromecambrine A (**57**) share the same basic skeleton with different substitution patterns.

Interestingly, the proaporphine litsericinone (**55**), showed the highest potency against the HepG2 cell line (IC_{50} = 14 μg/mL) due to the presence of a methylenedioxy and a carbonyl group at the C10 position (Likhitwitayawuid *et al*., 1993; Mohammed *et al*., 2012). The enhanced cytotoxic activity of compound hexahydromecambrine A (**57**) against the HepG2 cell line implies the presence of a 10-hydroxyl in the molecule was generally results in better potency than a non-hydroxylated proaporphine. Similarly, the weak cytotoxic activity displayed by compound 8,9,11,12-tetrahydromecambrine (**56**) compared to compound litsericinone (**55**), may be due to the presence of the *N*-methyl. These findings also suggested that the aromatic ring system, 7-oxo function, and methylenedioxy ring as well as the structural planarity have powerful effects on the cytotoxic activity against different cancer cell lines (Makarasen *et al.*, 2011).

The results shown in Table 4.25 implied that the cytotoxic property were related to compounds with 7-oxo and methylenedioxy functions, which corresponds to the result previously described by (Likhitwitayawuid *et al.,* 1993) that aporphine alkaloids containing a 1,2-methylenedioxy substituent appear to play a similar role in the oxoaporphine alkaloids, for the expression of cytotoxicity of the compounds.

4.8.2 Antibacterial activity

One known compound, namely lysicamine (**54**), and two new isolated compounds, namely litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**) were evaluated for antibacterial activity using disc diffusion method as previously described. The following

bacterial strains were employed in the test; *Staphylococcus epidermidis* a clinically isolated strain-UMMC (gram-positive), *Staphylococcus aureus* S1434-IMR culture (gram-positive), *Bacillus subtilis* B145-IMR culture (gram-positive), *Pasteurella multocida* a clinically isolated strain-UMMC (gram-negative) and *Enterobacter cloacae* a clinically isolated strain-UMMC (gram-negative).

The preliminary screening results of antibacterial activity against five bacterial species are summarised in Table 4.26. Antibacterial activities were indicated by a clear zone of growth inhibition. A standard antibiotic (streptomycin sulfate) was used as a positive control in the assay. The results indicated that lysicamine (**54**) exhibited a broad spectrum activity against the microbes compared to the other two compounds - litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**), which were found to be inactive.

Table 4.26: The diameter of inhibition zone (in mm) of isolated compounds from *phoebe grandis* leaves against selected bacteria.

Sample	Inhibition diameter (mm ± SD)				
	S. epidermidis	*S. aureus*	*B. subtilis*	*P. multocida*	*E. cloacae*
	(Gram +ve)	(Gram +ve)	(Gram +ve)	(Gram -ve)	(Gram -ve)
Lysicamine (**54**)	12.00 ± 0.00	13.33 ± 0.57	15.5 ± 0.57	NI	NI
Litsericinone (**55**)	NI	NI	NI	NI	NI
8,9,11,12-tetrahydromecambrine (**56**)	NI	NI	NI	NI	NI
Streptomycin sulphate [a]	20.00 ± 0.00	13.66 ± 0.57	21.00 ± 0.00	21.33 1.15	NI

Note : (NI) – no inhibition observed.
Doses of the sample were 1 mg/mL per disc.
Streptomycin sulphate 10 µg per disc.
[a] – positive control.

Lysicamine (**54**) displayed a strong antibacterial activity against *Staphylococcus aureus* with inhibition zones of 13.33 ± 0.57 mm, which are comparable with those of the positive control streptomycin sulfate. Moderate antibacterial activity was observed for lysicamine (**54**) against *Staphylococcus epidermidis* and *Bacillus subtilis*, with inhibition zones of 12.00 ± 0.00 mm and 15.50 ± 0.57 mm, respectively. *Pasteurella multocida* and *Enterobacter cloacae* (both Gram-negative bacteria) were not susceptible to any of the isolated compounds. Generally, the results showed that lysicamine (**54**) displayed strong activity against Gram-positive bacteria. The negative results obtained against the Gram-negative bacteria were not surprising, as in general, these bacteria are more resistant than Gram-positive ones (Vlietinck *et al*., 1995; Martin, 1995; Paz *et al*., 1995). These differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative cell wall is a multi-layered structure and quite complex (Yao, 1995; Nikaido, 1996) with an outer membrane consisting of a hydrophilic surface rich in lipopolysaccharide molecules (Gao *et al.*, 1999). In addition, the periplasmic space contains enzymes able to degrade any exogenous molecules and also prevent the entry of inhibitors, including antibiotic molecules (Martin, 1995; Duffy & Power, 2001). Gram-positive bacteria do not possess this type of outer membrane and cell wall structure. Therefore, antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and produce a leakage of the cytoplasm and its coagulation (Westh *et al*., 2004). The other two compounds litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**) were not toxic towards any of the tested pathogenic bacteria since no appreciable zones of inhibition were observed. The present results regarding the antibacterial properties of the compounds from leaves of *Phoebe grandis* (Nees) Merr. are potential to be used against Gram-positive pathogens. Their potential activity is probably due to their ability to complex with extracellular and soluble proteins and bacterial cell walls.

4.8.3 Antiplasmodial activity

Seven isolated compounds from CH_2Cl_2 extract of *P. tavoyana* leaves were tested for *in vitro* inhibitory activity against *P. falciparum* 3D7. Only four of the compounds resulted in significant inhibition activity against a chloroquine sensitive strain of *P. falciparum* (3D7); inhibitory concentration ranged from 0.89 - 2.76 μg/mL. They are summarised in Table 4.27 and Table 4.28. Among the compounds, roemerine (**20)** had the most potent inhibitory activity with IC_{50} value of 0.89 μg/mL. The well-known antimalaria drug, chloroquine has IC_{50} value of 0.006 μg/mL. The enhanced activity of roemerine (**20)** implies that the 1,2-methylenedioxy substituent is required for the expression of inhibitory activity of the aporphine alkaloids. The weak activity of norboldine (**6**) or laurolitsine (**6**) may be due to the absence of the 1,2-methylenedioxy or due to polarity which attenuated the ability to inhibit. This result supported previous studies that reported by Likhitwitayawuid *et al*., (1993), most of the aporphine alkaloids displayed significant antimalarial activity. The 1,2-methylenedioxy substituent appears to play a similar role in the oxo-aporphine alkaloids to enhance the antimalarial activity (Likhitwitayawuid *et al*., 1993).

It is important to notice that compounds boldine (**5**) and norboldine (**6**) share the same basic skeleton with different substitution of functional group N-Me and N-H. With regard to the structure of these compounds, boldine (**5**) which has only one N-Me group; while norboldine (**6**) with its N-H counterpart, was more active against *P. falciparum* 3D7 as compared to the N-Me derivative with the IC_{50} values 1.49 and 1.65 μg/mL, respectively. These data also support the previous study of another group of researcher Sudhanshu *et al*., (2003), reported that for each pair of 2-*nor*-alkaloid and its di-*N*-Me counterpart, the 2-*nor*-alkaloid which has only one Me group on the 2'-*N*, was

always more active against both susceptible and resistant strains of *Plasmodium* as compared to the di-N-Me derivative. For example, (+)-2-nor-thalrugosine was more active than those of (+)-thalrugosine (Saxena *et al*., 2003).

Unfortunately, laetanine (**62**), tavoyanine A (**63**) and tavoyanine B (**64**) were not tested due to insufficient amount to assessed the inhibitory *in vitro* activity against *P. falciparum*. In this investigation, we can imply that the IC_{50} values of laetanine (**62**) and norboldine (**6**) will be slightly the same since a similar structure-active relationship (SAR) was observed between laetanine (**62**) and norboldine (**2**). It might be due to each structure of the compounds has two methoxyl and two hydroxyl group at different position, and one N-H group.

Another interesting antimalarial compound was sebiferine (**22**), also found active against *in vitro P. falciparum* 3D7, but it was the weakest among the tested compounds. It may be due to the presence of the *N*-methyl and more methoxyl groups in the structure of sebiferine (**22**) compared to the others (Saxena *et al*., 2003).

Table 4.27: Inhibition Growth Percentage of *Plasmodium falciparum* and Probit Analysis with SPSS 11.5.

Sample	% Inhibition at Concentration (μg/mL)					IC_{50} (μg/mL)
	10	1	0.1	0.01	0.001	
Norboldine (**6**)	100.00	23.44	3.94	0.61	0.00	1.49
Boldine (**5**)	93.12	24.17	6.16	2.28	0.00	1.65
Laetanine (**62**)	-	-	-	-	-	nt

Note: Positive control is chloroquine, IC_{50} = 0.0069 μg/mL.
nt: not tested.

Table 4.28: Inhibition Growth Percentage of *Plasmodium falciparum* and Probit Analysis with SPSS 11.5.

Sample	% Inhibition at Concentration (μg/mL)					IC_{50} (μg/mL)
	100	10	1	0.1	0.01	
Roemerine (**20**)	99.81	99.30	36.10	15.23	0.95	0.89
Sebiferine (**22**)	100	94.72	6.60	1.05	0.66	2.76
Tavoyanine A (**63**)	-	-	-	-	-	nt
Tavoyanine B (**64**)	-	-	-	-	-	nt

Note: Positive control is chloroquine, IC_{50} = 0.0069 μg/mL.
nt: not tested.

Alkaloids are one of the major classes of compounds possessing antimalarial activity. In fact, one of the oldest and most important antimalarial drugs, quinine, belongs to this class of compounds and still relevant.

To the best of our knowledge, there has been no previous phytochemical investigation and medicinal value has been performed on this plant. These alkaloids norboldine (**6**), boldine (**5**), roemerine (**20**) and sebiferine (**22**) have no previous record of any antiplasmodial activity. The significant inhibitory activity of these alkaloids supports the traditional use of the *Phoebe* plants as an antimalarial drug.

CHAPTER 5

CONCLUSION

The phytochemical investigation of dichloromethane extract (acid-base) from leaves of *P. grandis* has resulted in the isolation of several common compounds including oxoaporphine and proaporphine alkaloids, such as lysicamine (**54**), dicentrinone (**58**), litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56**) and hexahydromecambrine A (**57**). These compounds are being reported for the first time in this species, while litsericinone (**55**) and 8,9,11,12-tetrahydromecambrine (**56**) were isolated also for the first time as natural compounds. Six compounds were isolated from the bark of *P. grandis* including two sterols and four aporphines. They were β-sitosterol (**59**) and stigmasterol (**60**); and boldine (**5**), N-methyllaurotetanine (**51**), reticuline (**61**) and laurolitsine **(6).**

Phytochemical study on the leaves of *P. tavoyana* afforded two new aporphines, tavoyanine A (**63**), tavoyanine B (**64**), together with four known aporphines; laurolitsine (**6),** roemerine (**20)**, laetanine (**62),** boldine (**5**) and one morphinandienone type, sebiferine (**22)**.

From the previous phytochemical search, *A. sesquipedalis* yielded only dicentrine (**52**) from the stem bark of the plant. However, *A. sesquipedalis* leaves and fruits parts have not been investigated before and afforded seven aporphines and two sterols. Six aporphines and two sterols were isolated from the leaves and three aporphines and two sterols isolated from the fruits. From the leaves aporphines dicentrine (**52**), N-

methyllaurotetanine (**51**), dicentrinone (**58**), boldine (**5**), norisocorydine (**65**), laurolitsine (**6**) and two sterols; β-sitosterol (**59**) and stigmasterol (**60**) were obtained. Meanwhile, the fruits afforded dicentrine (**52**), liriodenine (**19**), dicentrinone (**58**) and two sterols; β-sitosterol (**59**) and stigmasterol (**60**). Dicentrine (**52**) was a major compound isolated from *A. sesquipedalis,* while the second major isolated compound was dicentrinone (**58**). These are important for the chemotaxonomy of Lauraceae family.

The results indicated that a number of extracts present significant activities, such as cytotoxicity, antioxidant, antibacterial and antiplasmodial activities. This study provides valuable and useful information and indications for further exploring the potential nutraceutical and pharmaceutical applications of the Lauraceae tree species.

The significant *in vitro* anticancer activity assay results obtained against several human hepatocarcinoma (HepG2), human estrogen receptor (ER+) positive breast cancer (MCF7- Michigan Cancer Foundation-7) and human ovarian cancer cell line (Caov-3). Dichloromethane extract from leaves of *A. sesquipedalis*, was less sensitive towards MCF7 cell line with the IC_{50} value 30.00 µg/mL, but did not showed any activity against Caov-3 and HepG2 cell lines. Other extracts that displayed moderate activity against MCF7 cells were the dichloromethane extract (acid-base) from bark of *P. tavoyana*, dichloromethane extract (acid-base) from bark of *P. grandis* (KL 4994) and chloroform extract (acid-base) from bark of *P. tavoyana* with IC_{50} values 40.00, 45.00 and 48.00 µg/mL, respectively. The dichloromethane extract (acid-base) from bark of *P. tavoyana* also showed moderate cytotoxic effect towards Caov-3 with the IC_{50} value 35.00 µg/mL, while the dichloromethane extract (acid-base) from bark of *P. grandis* (KL 4994) gave the IC_{50} value of 45.00 µg/mL. All of the crude extracts did not show

any activity against HepG2, except dichloromethane extract from fruits of *A. sesquipedalis*, exhibited weak activity with IC_{50} value of 80.00 µg/mL.

However, an important observation that can be drawn from this work is that the *Actinodaphne* species were less sensitive towards MCF7 cell lines. It can also be concluded that at least one of the extracts in each species showed significant activity towards the selected assays.

While, for the isolated compounds lysicamine (**54**) demonstrated moderate activity against MCF7 and HepG2 cell lines with the IC_{50} value 26 and 27 µg/mL, respectively. Meanwhile litsericinone (**55**), 8,9,11,12-tetrahydromecambrine (**56)** and hexahydromecambrine A (**57**) showed weak cytotoxicity against MCF7 cell lines, but showed moderate activity against HepG2 with the IC_{50} value 14, 81 and 20 µg/mL, respectively.

None of the crude extracts of *P. grandis*, *P. tavoyana* and *A. sesquipedalis* exhibited notable DPPH free radical scavenging activity with the IC_{50} value > 500 µg/mL; except (acid-base) dichloromethane extract from *P. grandis* (bark) was weak activity observed with the IC_{50} value of 486.8 µg/mL. From the FRAP values, dichloromethane (acid-base) fraction of *P. tavoyana* (bark) showed value (3557.94 $\pm$ 0.07) had the highest ferric ions reducing activity and this potent antioxidant properties of *P. tavoyana* has not been reported before. Methanol extract of *A. sesquipedalis* (bark) showed good FRAP value (3128.78 $\pm$ 0.06) may be ascribed partially to the presence of high phenolic and flavonoid contents. Hexane extract of *P. tavoyana* (bark) displayed the lowest reducing power and all of n-hexane fractions were found to be poor activity compared to other extracts.

Twenty plant extracts from *Phoebe* and *Actinodaphne* species demonstrated varying levels of activity against six common bacteria. They are *Bacillus subtilis* B145-IMR culture (gram-positive), *Staphylococcus aureus* S1434-IMR culture (gram-positive), *Staphylococcus epidermidis* a clinically isolated strain-UMMC (gram-positive), *Escherichia coli* ATCC 25922 (gram-negative), *Salmonella typhi* clinically isolated strain-UMMC (gram-negative) and Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC BAA-1720 (gram-positive). Five alkaloids were identified from dichloromethane extract of *P. grandis* leaves, with lysicamine (**54**) being the most active compound with broad-spectrum antibacterial properties against *Staphylococcus aureus* with inhibition zones of 13.33 ± 0.57 mm, comparable with positive control streptomycin sulfate. Moderate antibacterial activity was observed for lysicamine (**54**) against *Staphylococcus epidermidis* and *Bacillus subtilis*, with inhibition zones of 12.00 ± 0.00 mm and 15.50 ± 0.57 mm, respectively. Therefore, further studies involving mechanisms of action are necessary to be carried in future to fully understand its biological significance.

Several aporphines of *P. tavoyana* strongly inhibited *in vitro* growth of a chloroquine sensitive strain of *Plasmodium falciparum* (3D7), with the strongest inhibition shown by roemerine (**20).** The structure-active relationship (SAR) of the tested compounds that exhibited significant antimalarial activity has been discussed. The antiplasmodial data with the IC_{50} value ranged from 0.89 - 2.76 μg/mL suggested that the leaves extract of *P. tavoyana* is a potential source of new antiplasmodial agents and the search for additional antimalarials from various type of plants must be continue to fight the disease. Interestingly, this is the first report for the antimalarial study for this plant.

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LIST OF PUBLICATIONS

ISI Journals

1. Noraziah Nordin, Suzy Munir Salama, Shahram Golbabapour, Maryam Hajrezaie, Pouya Hassandarvish, Behnam Kamalidehghan, Nazia Abdul Majid, Najihah Mohd Hashim, **Hanita Omar**, Mehran Fadaienasab, Hamed Karimian, Hairin Taha, Hapipah Mohd Ali, Mahmood Ameen Abdulla.
   Anti-Ulcerogenic Effect of Methanolic Extracts from Enicosanthellum pulchrum (King) Heusden against Ethanol-Induced Acute Gastric Lesion in Animal Models.
   PLOS ONE. November 2014 | Volume 9 | Issue 11 | e111925. **Published November 7, 2014.**

2. Noraziah Nordin*, Syam Mohan, Najihah Mohd. Hashim, Asdren Zajmi, Noor Shafifiyaz Mohd. Yazid, Mashitoh Abdul Rahman, Fatima Abdelmutaal Ahmed Omer, **Hanita Omar,** Fatimah A'fifah Alias & Hapipah Mohd. Ali
   Antioxidant, Anticancer and Antimicrobial Activities of Methanolic Extracts from *Enicosanthellum pulchrum* (King) Heusden.
   SAINS MALAYSIANA 43(10)(2014): 1515–1521. **Published**.

3. Berna Elya, Roshamur C. Forestrania, Mat Ropi, Soleh Kosela, Khalijah Awang, **Hanita Omar** and A. Hamid A. Hadi.
   The New Alkaloids from *Antidesma cuspidatum* M.A.
   Rec. Nat. Prod. 8:4(2014) 342-347. **Published**.

4. **Hanita Omar***, Najihah Mohd. Ali, Asdren Zajmi, Noraziah Nordin, Siddiq Ibrahim Abdelwahab, Ainnul Hamidah Syahadah Azizan, A. Hamid A. Hadi and Hapipah Mohd. Ali.
   Aporphine Alkaloids from the Leaves of *Phoebe grandis* (Nees) Mer. (Lauraceae) and Their Cytotoxic and Antibacterial Activities.
   (***Molecules***, 2013, 18, 08994) **Published**.

Manuscript in preparation

1. **Hanita Omar***, Aty Widyawaruyanti, Mohd Azlan Nafiah, Tiah Rachmatiah, Mehran Fadaeinasab, Ainnul Hamidah Syahadah Azizan, Najihah Mohd. Hashim, A. Hamid A. Hadi, Hapipah Mohd Ali .
   In Vitro Antiplasmodial Activity of Aporphine Alkaloids Isolated from leaves of *Phoebe tavoyana* (Meissn.) H.k.F. ***Phytochemistry Letter***.

2. **Hanita Omar***, Shahram Golbabapour, Pouya Hassandarvish, Maryam Hajrezaie, Ainnul Hamidah Syahadah Azizan, Noraziah Nordin, Mehran Fadaei Nasab, Nazia Abdul Majid, Mahmood Ameen Abdulla, Najihah Mohd. Hashim, Hapipah Mohd. Ali.
   Methanol Extract of *Actinodaphne sesquipedalis* (Lauraceae) enhance gastric defence against ethanol-induced gastric ulcer in mice. **PLOS ONE**.

Papers and posters presented at seminars and exhibitions

1. Alkaloids isolated from the leaves of *Phoebe tavoyana* (Lauraceae) and their antiplasmodial activity.
   Hanita Omar*, Najihah Mohd. Hashim, A. Hamid A. Hadi, Hapipah Mohd Ali.
   International Conference on Natural Products 2014.
   Palm Garden Hotel, Putrajaya, Malaysia.
   18-19th March 2014. -***Poster Presentation.***

2. Antibacterial activity of oxoaporphine alkaloid compound against MDR *Staphylococcus aureus*.
   Asdren Zajmi*, Najihah Mohd. Hashim, **Hanita Omar**, Mohamed Ibrahim Nordin and Hapipah Mohd Ali
   International Conference on Natural Products 2014.
   Palm Garden Hotel, Putrajaya, Malaysia.
   18-19th March 2014. -***Poster Presentation***.

3. Cytotoxic and antibacterial activities from the leaves of *Phoebe grandis* (Nees) Mer. (Lauraceae).
   Hanita Omar, Najihah Mohd. Hashim, Asdren Zajmi, A. Hamid A. Hadi, Hapipah Mohd Ali.
   14th Tetrahedron Symposium Asia Edition, Seoul, Republic of Korea.
   21-24th Oct 2013. -***Poster Presentation***.

4. Antibacteria and Antioxidant Activities from the Extracts of the Leaves of *Phoebe Grandis* (Ness) Merr.
   Hanita Omar, Noor Shafifiyaz Mohd Yazid, Asdren Zajmi, Najihah Mohd. Hashim, A. Hamid A. Hadi
   International Conference on Natural Products 2013 (ICNP 2013), Shah Alam Convention Centre (SACC), Selangor, Malaysia.
   4-6th March 2013. -***Poster Presentation.***

5. HMBC spectra of alkaloids from Lauraceae Species:Nine Stars Halo-N Theories
   O. Hanita, A. H. A. Hadi, Ahmad Laksamana Omar
   4th Regional Conference on Natural Resources in the Tropics (NTrop4),
   DeTAR PUTRA, UNIMAS, Kota Samarahan, Sarawak.
   19th – 21st September 2012. – ***Oral presentation.***

6. Aporphine alkaloids and Antioxidant Activity of *Phoebe grandis* (Nees) Merr. –
   O. Hanita, M.M. Azrul, A. H. A. Hadi
   4th Regional Conference on Natural Resources in the Tropics (NTrop4),
   DeTAR PUTRA, UNIMAS, Kota Samarahan, Sarawak.
   19th – 21st September 2012. - ***Oral presentation.***

7. Alkaloids from the bark of *Phoebe tavoyana*
   Hanita Omar, Anissuhailin Zainal abidin, Wan Nurul Naznem Wan Othman, A. Hamid A. Hadi.
   One-Day Scientific & Motivation Seminar 2010.
   Crystal Crown Hotel, Petaling Jaya, Malaysia.
   22nd December 2010. - ***Poster Presentation.***

8. The Application Of The Nine Stars Halo-N Theory In The Natural Product Research.
   Hamid A. Hadi, **Hanita Omar** and Ahmad Laksamana Omar
   (Proceeding – Malaysian Journal of Pharmaceutical Sciences).
   Title: International Conference on Natural Products 2010 (ICNP).
   10th-12th December 2010,
   Bayview Beach Resort, Penang. - ***Oral Presentation***.

9. Antiplasmodial from *Phoebe tavoyana*
   Hanita Omar, Berna Elya, Mohd Azlan Nafiah, Khalijah Awang, Mat Ropi Mukhtar and A.Hamid A.Hadi.
   (Proceeding-SciFinder).
   Kamphaengsaen International Natural Products Symposium: The Relationship between Living Organisms and Environment, Proceeding Book, 1st, Bangkok, Thailand.
   Oct. 23-24, 2010 (2010), 191-202. - ***Oral Presentation***.

10. New N-2-hydroxypropyl aporphines from Lauraceae species
    A.Hamid A.Hadi, Tiah Rachmatiah, **Hanita Omar**, Mohd Azlan Nafiah, Mat Ropi Mukhtar, and Khalijah Awang.
    (Proceeding-SciFinder).
    Kamphaengsaen International Natural Products Symposium: The Relationship between Living Organisms and Environment, Proceeding Book, 1st, Bangkok, Thailand.
    Oct. 23-24, 2010 (2010), 191-202. - ***Oral Presentation***.

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Anti-Ulcerogenic Effect of Methanolic Extracts from *Enicosanthellum pulchrum* (King) Heusden against Ethanol-Induced Acute Gastric Lesion in Animal Models

Noraziah Nordin[1,3], Suzy Munir Salama[2]*, Shahram Golbabapour[2,4], Maryam Hajrezaie[2,4], Pouya Hassandarvish[2], Behnam Kamalidehghan[1], Nazia Abdul Majid[4], Najihah Mohd Hashim[1], Hanita Omar[3,5], Mehran Fadaienasab[3], Hamed Karimian[1], Hairin Taha[3], Hapipah Mohd Ali[3], Mahmood Ameen Abdulla[2]*

1 Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, 2 Department of Biomedical Sciences, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, 3 Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, 4 Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia, 5 Center of Foundation Studies in Sciences, University of Malaya, Kuala Lumpur, Malaysia

Abstract

A natural source of medicine, *Enicosanthellum pulchrum* is a tropical plant which belongs to the family Annonaceae. In this study, methanol extract from the leaves and stems of this species was evaluated for its gastroprotective potential against mucosal lesions induced by ethanol in rats. Seven groups of rats were assigned, groups 1 and 2 were given Tween 20 (10% v/v) orally. Group 3 was administered omeprazole 20 mg/kg (10% Tween 20) whilst the remaining groups received the leaf and stem extracts at doses of 150 and 300 mg/kg, respectively. After an additional hour, the rats in groups 2–7 received ethanol (95% v/v; 8 mL/kg) orally while group 1 received Tween 20 (10% v/v) instead. Rats were sacrificed after 1 h and their stomachs subjected to further studies. Macroscopically and histologically, group 2 rats showed extremely severe disruption of the gastric mucosa compared to rats pre-treated with the *E. pulchrum* extracts based on the ulcer index, where remarkable protection was noticed. Meanwhile, a significant percentage of inhibition was shown with the stem extract at 62% (150 mg/kg) and 65% (300 mg/kg), whilst the percentage with the leaf extract at doses of 150 and 300 mg/kg was 63% and 75%, respectively. An increase in mucus content, nitric oxide, glutathione, prostaglandin E2, superoxide dismutase, protein and catalase, and a decrease in malondialdehyde level compared to group 2 were also obtained. Furthermore, immunohistochemical staining of groups 4–7 exhibited down-regulation of Bax and up-regulation of Hsp70 proteins. The methanol extract from the leaves and the stems showed notable gastroprotective potential against ethanol.

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* Email: s.salama999@hotmail.com (SMS); ammeen@um.edu.my (MAA)

Introduction

Stomach ulcer is a gastric mucosal rupture that extends via the muscularis mucosa into the submucosa or deeper [1]. For decades, this disease has been considered one of the world's most common illnesses, and it is a global problem among youths [2]. Some of the main aggressive factors that can contribute to ulcer are gastric acid, pepsin, bile salts, abnormal motility, alcohol and nonsteroidal anti-inflammation drugs (NSAID), as well as infection with *Helicobacter pylori*. However, there are several factors that can protect the stomach from ulcer formation such as mucus secretion, gastroprotective prostaglandin synthesis, bicarbonate production and normal tissue microcirculation [3,4,5,6]. Reducing gastric acid production and increasing gastric mucosal protection have been the major strategies proposed for the prevention of peptic ulcer disease [7]. Ethanol-induced gastric injury is a commonly known method used in the evaluation of therapeutic potencies against gastric ulcer [8]. Ethanol causes intense lesions penetrating into the submucosa [9], enhances reactive oxygen species (ROS) formation, and depletes the mucus membrane [10,11,12] which renders cell death in gastric mucosal cells. Ethanol also inhibits cyclooxygenase enzyme and suppresses the output of endogenous prostaglandins [13].

Alternative and complementary medicine has gained global attention because of their widespread use in the field of medicine for treating many diseases. There are three species of the Annonaceae family which also have antiulcer activities such as *Polyalthia longifolia* cv. Pendula. [14], *Annona squamosa* L. [15] and *Annona reticulata* L. [16]. Some of these have been traditionally used to treat peptic ulcers. The protective potential of many other compounds and extracts have been evaluated according to how they enhance gastric toleration against aggressive factors such as endogenous, exogenous or infectious agents. Studies on synthetic compounds and plant extracts emphasize the importance of introducing novel herbal resources with gastroprotective healing properties.

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Antioxidant, Anticancer and Antimicrobial Activities of Methanolic Extracts from *Enicosanthellum pulchrum* (King) Heusden

(Aktiviti Antioksidan, Antikanser dan Antimikrobial Ekstrak Kasar *Enicosanthellum pulchrum* (King) Heusden)

NORAZIAH NORDIN*, SYAM MOHAN, NAJIHAH MOHD. HASHIM, ASDREN ZAJMI, NOOR SHAFIFIYAZ MOHD. YAZID, MASHITOH ABDUL RAHMAN, FATIMA ABDELMUTAAL AHMED OMER, HANITA OMAR, FATIMAH A'FIFAH ALIAS & HAPIPAH MOHD. ALI

ABSTRACT

Biological activities of crude methanolic extracts from leaves, barks, twigs and roots of Enicosanthellum pulchrum *were investigated in four bioassays. The antioxidant, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay showed that bark and twig extracts showed high inhibitory activity with 60 and 56% inhibition at 1 mg/mL and* IC_{50} *values of 0.43 ± 0.04 and 0.64 ± 0.05 mg/mL, respectively. The bark and root extracts showed greater reducing power (FRAP) than several standard drugs used in the bioassay. Methanolic extracts of leaves, twigs and roots displayed strong cytotoxicity to breast cancer cell line (MCF-7), myelomonocytic leukaemia cell line (WEHI-3) and ovarian cancer cell line (CAOV-3); the* IC_{50} *of the leaf extract were 7.8 ± 0.85 µg/mL (MCF-7) and 9.0 ± 0.13 µg/mL (WEHI-3), while those for the twig and root extracts were 13.9 ± 0.35 and 7.3 ± 0.98 µg/mL (CAOV-3), respectively. In the antimicrobial assays, the extracts were tested against ten bacterial strains and two fungal strains. Bark and twig extracts displayed high inhibitory activity to* Bacillus subtilis *with 13.3 ± 0.57 and 12.0 ± 0.01 mm inhibition, respectively. In addition, the twig extract displayed better minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) compared with the bark extract (MIC 0.5 and 1.0 mg/mL, MBC 1.0 and 2.0 mg/mL, respectively). For antifungal activity, all extracts showed inhibition on* Candida albicans *but not on* Aspergillus niger. *The obtained results suggested that this plant may possibly contain bioactive compounds in the active extracts.*

Keywords: Anticancer; antimicrobial; antioxidant; Enicosanthellum pulchrum*; methanolic extracts*

ABSTRAK

Aktiviti biologi Enicosanthellum pulchrum *bagi ekstrak kasar metanol daun, kulit, batang dan akar telah dikaji terhadap empat bioasai. Asai skaveng antioksidan, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) mendedahkan bahawa ekstrak kulit dan batang menunjukkan aktiviti perencatan yang tinggi dengan perencatan sebanyak 60 dan 56% pada kepekatan 1 mg/mL, manakala nilai* IC_{50} *masing-masing adalah 0.43 ± 0.04 dan 0.64 ± 0.05 mg/mL. Ekstrak kulit dan akar menunjukkan kuasa pengurangan yang lebih besar (FRAP) berbanding dadah kawalan yang digunakan dalam bioasai ini. Ekstrak metanol daun, batang dan akar memaparkan sitotoksisiti yang kuat terhadap titisan sel kanser payudara (MCF-7), leukimia milomonositik (WEHI-3) dan kanser ovari (CAOV-3); nilai* IC_{50} *bagi ekstrak daun 7.8 ± 0.85 µg/mL (MCF-7), manakala ekstrak batang dan akar masing-masing adalah 13.9 ± 0.35 dan 7.3 ± 0.98 µg/mL (CAOV-3). Bagi asai antimikrob, ekstrak diuji terhadap sepuluh strain bakteria dan dua strain fungus. Ekstrak kulit dan batang memaparkan aktiviti perencatan yang tinggi terhadap bakteria* bacillus subtilis *dengan perencatan masing-masing sebanyak 13.3 ± 0.57 dan 12.0 ± 0.01 mm. Bacaan bagi kepekatan perencatan minimum (MIC) dan kepekatan bakterisidal minimum (MBC) bagi kedua-dua ekstrak menunjukkan ekstrak batang memberikan bacaan MIC dan MBC yang lebih baik berbanding ekstrak kulit dengan bacaan masing-masing sebanyak (MIC 0.5 dan 1.0 mg/mL, MBC 1.0 dan 2.0 mg/mL). Bagi aktiviti antifungus, semua ekstrak memberikan perencatan terhadap* Candida albicans *dan tidak pada* Aspergillus niger. *Keputusan yang diperoleh mencadangkan bahawa tumbuhan ini berkumungkinan mempunyai sebatian bioaktif daripada ekstrak aktif.*

Kata kunci: Antikanser; antimikrob; antioksidan; ekstrak metanol; Enicosanthellum pulchrum

INTRODUCTION

Natural products have been recognized as an important tool in the drug discovery process throughout this century (Agosta 1997; Sthrol 2000). Many studies have been conducted especially on plants in order to identify chemical compounds that can act as remedies for various diseases. In fact, plants have been reported to be used worldwide for medicinal purposes (Duke 2000). However, the search for biologically active compounds requires a bioassay procedure to detect a certain type of biological activity in the corresponding crude extract (Hostettmann et al. 1997). The development of biological activity studies on the plant extracts and compounds in Malaysia actively began in the early 90's (Jantan 2008). Some specific

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Noraziah Nordin*, Syam Mohan, Najihah Mohd Hashim, Asdren Zajmi, Noor Shafifiyaz Mohd Yazid, Mashitoh Abdul Rahman & Fatima Abdelmutaal Ahmed Omer
Department of Pharmacy, Faculty of Medicine
University of Malaya
50603 Kuala Lumpur
Malaysia

Hanita Omar, Fatimah A'fifah Alias & Hapipah Mohd Ali
Department of Chemistry, Faculty of Science
University of Malaya
50603 Kuala Lumpur
Malaysia

Hanita Omar
Centre for Foundation Studies in Sciences
University of Malaya
50603 Kuala Lumpur
Malaysia

*Corresponding author; email: aziereality@yahoo.com

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records of natural products

The New Alkaloids from *Antidesma cuspidatum* M.A.

Berna Elya[1], Roshamur C. Forestrania[1], Mat Ropi[2], Soleh Kosela[3], Khalijah Awang[2], Hanita Omar[2] and A. Hamid A. Hadi[2*]

[1]*Faculty of Pharmacy, University of Indonesia, Kampus UI Depok 16424, Indonesia*

[2]*Department of Chemistry, University Malaya, Kuala Lumpur, Malaysia*

[3]*Department of Chemistry, University of Indonesia, Kampus UI Depok 16424, Indonesia*

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Abstract: Two new alkaloids, cuspidatin (**1**) and cuspidatinol (**2**), were isolated from *Antidesma cuspidatum* M.A. (Euphorbiaceae). The structures were determined by means of spectroscopic analysis (IR, MS, and NMR spectroscopy). The results of cytotoxicity evaluation showed that compounds **1** and **2** were inhibitory to L1210 cells, with IC_{50} values 8.41 and 6.36 µg/mL respectively.

Keywords: *Antidesma cuspidatum* M.A.; Alkaloids; Euphorbiaceae; Cytotoxic activity.

1. Introduction

Antidesma is a tropical plants genus classified in family Phyllanthaceae (Malpighiales; Euphorbiaceae sensu lato). Most of the genus in South-East Asia is commonly found in the understorey of tropical forests as well as in open vegetation [1]. They have approximately 170 Antidesma species in the world including South-East Asia [2].

Antidesma cuspidatum M.A. is one of the species of Antidesma, which is largely distributed in Malaysia, Borneo, and Indonesia, especially in Sulawesi. The leaves of "Kenida punai", an Indonesia local name of *A. cuspidatum*, have been applied as traditional carminative medicine. The chemical research of genus Antidesma led to the isolation of new alkaloids. Recently, antidesmone, a bicyclic alkaloid, was isolated from Euphorbiaceae species of the East African plants, *A. membranaceum* Müll. Arg. and *A. venosum* E. Mey. Furthermore, belonging to this research, its structure has been revised into (S)-4,8-dioxo-3-methoxy-2-methyl-5-n-octyl-1,4,5,6,7,8-hexahydroquinoline [3]. The other research yield also report a discovery of other compounds indentified as alkaloids from Antidesma. [4,5,6]. These researches indirectly indicate a potential of *A. cuspidatum* as a species which is probably has alkaloid content as well as the other species of genus Antidesma reported.

Medicinal plants have been a useful source for the research of new biological active compounds. Apart from the medicinal effects of traditional herbs, exploratory researches have been made and a wide variety of new biological activities from traditional medicinal plants have recently been reported, including anticancer activity [7]. Based on scientific evidence of natural products in cancer therapy, now days, it had been known that natural resources are playing role of about 60% anticancer drugs [8]. For instance, cytotoxic activities were performed by the compounds from one of the species Antidesma. Four characterized alkaloid compounds of *A. theaitesiamnum* Müll. Arg.

*Corresponding author: E-Mail: berna.elya@farmasi.ui.ac.id; Phone: +628 138 3078647.

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exhibit cytotoxic effects in microgram per milliliter concentration range, with IC_{50} value 0.85, 0.78, and 0.95 µg/mL respectively. All of these compounds used can decrease in intracellular reactive oxygen species resulting in an increase percentage of early apoptotic cells [9]. The facts provide a probability of another species of genus Antidesma to extend cytotoxic activity related antioxidative properties of their alkaloid compound, including alkaloid compound of *A. cuspidatum*. Here we report a structure of alkaloid compound within bark of *A. cuspidatum* as a pilot study of this species. Isolated compound then applied to perform cytotoxicity assay using L1210 murine leukemia cell line. The activity was represented as IC_{50} (the concentration at which growth or activity is inhibited by 50% in certain period).

2. Materials and Methods

2.1. General

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in deuterated chloroform on a JEOL 400 MHz instrument. Silica gel 60, 70–230 mesh ASTM (Merck 7734) was used for column chromatography. Mayer's reagent was used for alkaloid screening. TLC aluminium sheets (20 × 20 cm Silica gel 60 F_{254}) were used in the TLC analysis. The TLC spots were visualized under UV light (254 and 366 nm) followed by spraying with Dragendorff's reagent for an alkaloid detection.

2.2 Plant Material

The bark of *A. cuspidatum* (Euphorbiaceae), collected from Simpan Sungai Badak Forest, Sintok, Kedah, Malaysia in April 26th 2006, was identified by Mr Teo Leong Eng and Din. A voucher specimen (KL 5230) is deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia and at the Herbarium of the Forest Research Institute, Kepong, Malaysia.

2.3 Extraction and Isolation of the Alkaloid

The dried bark (2.5 kg) of *A. cuspidatum* (Euphorbiaceae) were extracted with hexane (25.0 *l*) for 18 hours. The residual plant material was dried and left for 2 h after moistening with 25% NH_4OH. Then, It macerated with CH_2Cl_2 (25.0 *l*) twice for 3-days. The extract CH_2Cl_2 was concentrated to give residue (7,0 g) that was subjected to column chromatography on silica gel (column dimension = 3 cm, length = 1 m, silica gel 60, 70–230 mesh ASTM; Merck 7734) using CH_2Cl_2 gradually enriched with methanol to yield 58 fractions. Fractions were then recombined on the basis of their TLC behavior to obtain 7 fractions. Fractions 4 (98 mg), afforded an alkaloid identified as cuspidatin (**1**), using PTLC (Merck KGaA silica gel 60 F_{254}; CH_2Cl_2-MeOH; 9:1). Cuspidatinol (**2**) was obtained from fraction 5 (198 mg) by using PTLC (Merck KGaA silica gel 60 F_{254}; CH_2Cl_2-MeOH; 8.5:1.5).

2.4 In Vitro Cytotoxicity Assay

Isolate **1** and **2** were tested its cytotoxic activity against L1210 mouse leukemia cells by *in vitro* technique. L1210 cells were provided by Natural Product PATIR BATAN Laboratory acquired from The Institute of Physical and Chemical Research (RIKEN) Japan. RPMI-1640 was used as media containing glutamine and sodium bicarbonate. A total of 15 mL calf bovine serum was added into 85 mL of medium. All activities were done in a steril room. A total of media containing 2 x 10^5 cells/mL were incorporated into every hole in tissue culture plate. The sample were serially diluted in dimethyl sulfoxide to produce 5 concentrations and each concentration was added to plates in 3 replicates to obtain final isolates concentration of 0 (control), 1, 2, 4, and 8 µg/mL. The plates were incubated for 48 h in 5% CO_2 incubator at 37°C, then the number of cells were counted using Neubauer haemocytometer improved under a microscope. The percentages of inhibition were calculated by comparison of viable cells in the samples with viable cells in the control. The tryptan blue exclusion method was used to determine cell viability. The inhibitory percentage of the test was calculated to

obtain IC_{50}. The IC_{50} value (*inhibitory concentration fifty*) is the concentration of sample that inhibits the proliferation of leukemia L1210 cells in 50% can be obtained from linear regression of logarithmic of test concentration and probit of inhibition percentage. The smaller the IC_{50} value of a test, the higher its cytotoxic activity.

3. Results and Discussion

3.1. Structure elucidation

Cuspidatin or 4-[2-(2-(dimethylamino) ethyl)-4,5-dimethoxyphenethyl]phenol (Figure 1), noticed as compound **1**, was obtained as a brownish amorphous solid. The LC-MS spectrum the molecular ion peak at *m/z* 330.2077 $[M + H]^+$, thus suggesting formula of $C_{20}H_{27}NO_3$. IR spectrum emerged strong absorption at 3383 cm^{-1} indicating of hydroxyl group. The ^{1}H-NMR spectrum (Table 1) showed one proton singlet at δ 6.60 in aromatic region which is characteristic of 1,3,4,6-tetrasubstituted phenyl ring (ring A), with two methoxyl group (6H, s, δ 3.81). Another set of 2 H doublets at δ_H 6.83 and 6,72 is due to the 1,4-disubstituted benzene ring system ($\alpha_2\beta_2$, $J_{\alpha\beta}$ = 8.3 Hz, H-2′, H-6′ and H-3′, H-5′ respectively). The remaining resonances were for the aliphatic protons H-α and H-β observed as multiplets (due to possible conformational preferences) at δ_H 3.15 and 2.89 respectively; H-α′ and H-β′ (overlapped as a broad singlet) at 2.79, two NMe groups (6H, s, δ 2.47) , and one hydroxyl group at δ_H 4.76. The ^{13}C-NMR spectrum of compound **1** (Table 1) shows signals of 12 aromatic carbons constituting the two phenil ring, two methoxyl group (δ_C 55.94 and 56.01 ppm) four methylene of which one carbon (C-α 59.60 ppm) is N-linked and two other NMe groups. The characterization above is confirmed by the HMBC spectrum (Figure 2).

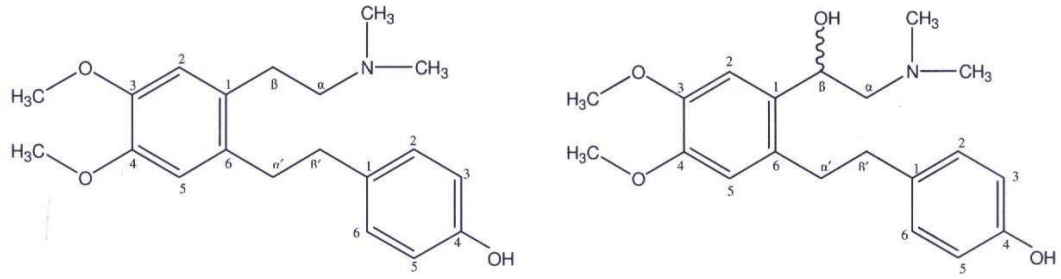

Figure 1. Structure of compounds **1** and **2** from *A. cuspidatum*.

Cuspidatinol or compound **2** (Figure 1) was isolated as a brownish amorphous solid with the molecular ion peak at *m/z* 346.2107 $[M + H]^+$ indicating formula of $C_{20}H_{27}NO_4$. IR spectroscopy data revealed the presence of hydroxyl at 3385 cm^{-1}. The comparison of ^{13}C-NMR and ^{1}H-NMR showed that the difference between compound **1** (Table 1) and **2** (Table 2) only at C-β of **1** which is replaced by hydroxyl group in compound **2**. Proton H-β revealed a singlet peak (torsional angle 90° to H-α). The correlation of the protons and carbons which sustained the characterization of compound **2** was mentioned in HMBC spectral data (Figure 2).

Table 1. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), and HMBC spectroscopic data of compound **1** ($CDCl_3$, δ in ppm, *J* in Hz).

Position	Chemical shift (δ) (ppm)		HMBC
	^{1}H-NMR	^{13}C-NMR	
1	-	129.65 (C)	-
2	6.60 (*s*)	112.86 (CH)	1, 3, β
3	-	147.72 (C)	-
4	-	147.28 (C)	-
5	6.60 (*s*)	112.73 (CH)	4, 6
6	-	127.88 (C)	-
β′	2.79 *	31.93 (CH_2)	α′, 6, 1′, 6′, 2′
α′	2.79 *	37.30 (CH_2)	β′, 6, 1′
β	2.89 *	34.66 (CH_2)	-
α	3.15 *	59.60 (CH_2)	-
1′	-	132.67 (C)	-
2′	6.83 (*d*, *J* = 8.3 Hz)	131.95 (CH)	1′, 4′
3′	6.72 (*d*, *J* = 8.3 Hz)	115.58 (CH)	1′, 2′, 4′
4′	-	155.15 (C)	-
5′	6.72 (*d*, *J* = 8.3 Hz)	115.58 (CH)	6′, 4′
6′	6.83 (*d*, *J* = 8.3 Hz)	131.95 (CH)	1′, 5′, 4′
NMe $_{(2)}$	2.47 (*sb*)	43.76 (CH_3)	-
3-OMe	3.81 (*s*)	55.94 (CH_3)	3
4-OMe	3.87 (*s*)	56.01 (CH_3)	4
4′-OH	4.76 (*s*)	-	-
β-OH	-	-	-

Asterisks (*) mark overlapping signals

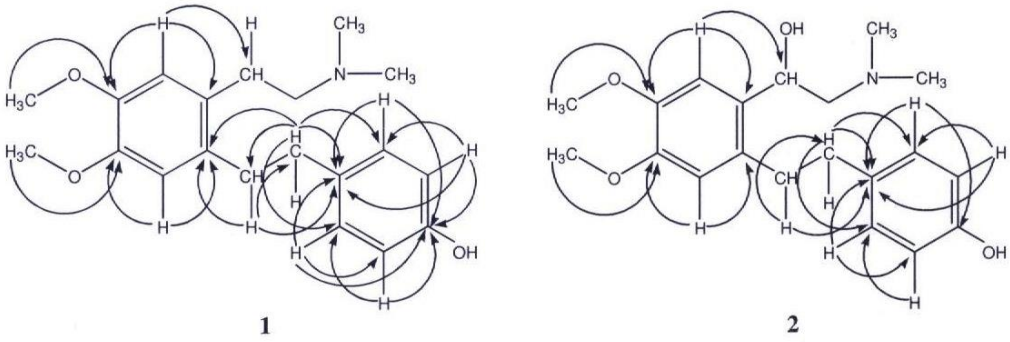

Figure 2. Key HMBC correlations (H → C) of compound **1** and **2**.

Table 2. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), and HMBC spectroscopic data of compound **2** ($CDCl_3$, δ in ppm, *J* in Hz).

Position	Chemical shift (δ) (ppm)		HMBC
	^{1}H-NMR	^{13}C-NMR	
1	-	129.81 (C)	-
2	6.61 (*s*)	112.99 (CH)	1, 3, β
3	-	147.62 (C)	-
4	-	148.31 (C)	-
5	6.61 (*s*)	112.99 (CH)	4, 6
6	-	126.14 (C)	-
β′	2.81 *	26.06 (CH_2)	1′, 6′ 2′
α′	2.81 *	29.79 (CH_2)	β′, 1′
β	3.16 *	37.49 (CH_2)	-
α	2.91 *	34.72 (CH_2)	-
1′	-	129.81 (C)	-
2′	6.82 (*d*, *J* = 8.3 Hz)	132.04 (CH)	1′, 4′
3′	6.71 (*d*, *J* = 8.3 Hz)	115.84 (CH)	1′, 2′
4′	-	156.27 (C)	-
5′	6.71 (*d*, *J* = 8.3 Hz)	115.84 (CH)	6′
6′	6.82 (*d*, *J* = 8.3 Hz)	132.04 (CH)	1′, 5′
NMe $_{(2)}$	2,44 *sb*	56.30 (CH_3)	-
3-OMe	3.82*s*	56.04 (CH_3)	3
4-OMe	3.88*s*	57.73 (CH_3)	4
4′-OH	4.75*s*	-	-
β-OH	4.75*s*	-	-

Asterisks (*) mark overlapping signals

3.2 Cytotoxic activity

The compound **1** and **2** were evaluated their cytotoxic activity against L1210 mouse leukemia cells by in vitro technique. The results of cytotoxicity assessment showed that compound **1** and **2** exhibit cytotoxicity with IC_{50} 8.41 and 6.36 µg/mL respectively.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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Article

Aporphine Alkaloids from the Leaves of *Phoebe grandis* (Nees) Mer. (Lauraceae) and Their Cytotoxic and Antibacterial Activities

Hanita Omar [1,2,*], Najihah Mohd. Hashim [3], Asdren Zajmi [3], Noraziah Nordin [3], Siddiq Ibrahim Abdelwahab [4], Ainnul Hamidah Syahadah Azizan [1], A. Hamid A. Hadi [1] and Hapipah Mohd Ali [1,*]

[1] Chemistry Department, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; E-Mails: ainnul_azizan@yahoo.com (A.H.S.A.); ahamid@um.edu.my (A.H.A.H.)

[2] Centre for Foundation Studies in Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia

[3] Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; E-Mails: najihahmh@gmail.com (N.M.H.); asza22@yahoo.com (A.Z.); aziereality@yahoo.com (N.N.)

[4] Medical Research Center, Faculty of Medicine, Jazan University, Jazan 45041, Saudi Arabia; E-Mail: siddigroa@yahoo.com

* Authors to whom correspondence should be addressed; E-Mails: hanita74@um.edu.my (H.O.); hapipah@um.edu.my (H.M.A.); Tel.: +603-7967-4246 (H.M.A.); Fax: +603-7967-4193 (H.M.A.).

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Abstract: The oxoaporphine alkaloid lysicamine (**1**), and three proaporphine alkaloids, litsericinone (**2**), 8,9,11,12-tetrahydromecambrine (**3**) and hexahydromecambrine A (**4**) were isolated from the leaves of *Phoebe grandis* (Nees) Merr. (Lauraceae). Compounds **2** and **3** were first time isolated as new naturally occurring compounds from plants. The NMR data for the compounds **2**–**4** have never been reported so far. Compounds **1** and **2** showed significant cytotoxic activity against a MCF7 (human estrogen receptor (ER+) positive breast cancer) cell line with IC_{50} values of 26 and 60 μg/mL, respectively. Furthermore, *in vitro* cytotoxic activity against HepG2 (human liver cancer) cell line was evaluated for compounds **1**–**4** with IC_{50} values of 27, 14, 81 and 20 μg/mL, respectively. Lysicamine (**1**) displayed strong antibacterial activity against *Bacillus subtilis* (B145), *Staphylococcus aureus* (S1434) and *Staphylococus epidermidis* (a clinically isolated strain) with inhibition zones of 15.50 ± 0.57, 13.33 ± 0.57 and 12.00 ± 0.00 mm, respectively.

However, none of the tested pathogenic bacteria were susceptible towards compounds **2** and **3**.

Keywords: *Phoebe grandis*; Lauraceae; aporphine alkaloid; cytotoxic; antibacterial

1. Introduction

Cancer is the most common and fatal disease and accounted for 7.6 million deaths (about 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 [1]. Breast cancer is one of the main life-treatening diseases that a woman may have to face during her lifetime and is the most widespread [2].

About 60% of anticancer drugs used nowadays are obtained from natural sources [3]. Out of total 250,000 plant species existing on Earth approximately one thousand have known anticancer potential. A large number of plant species have been screened through bioassays in the search for novel plant-based anticancer drugs [4]. The present investigation aimed to assess the cytotoxic potential of alkaloids isolated from leaves of *Phoebe grandis* (Nees) Merr. (Lauraceae) on selected cell lines.

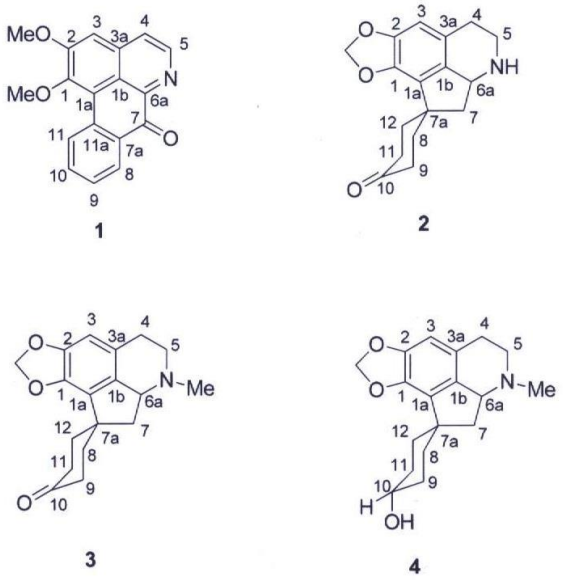
Over the last decade, antibiotics which were considered to be miracle drugs have been losing their effectiveness as pathogens evolve resistance against them [5]. Antibacterial resistance continues to grow quickly among key pathogens such as *Staphylococcus epidermidis* (a Gram-positive bacterium)—which is a part of human skin flora and also can be found in the mucous membranes and in animals [6]; *Staphylococcus aureus* (a Gram-positive bacterium) is frequently found in the human respiratory tract and on the skin, and it is estimated that 20% of the human population are long-term carriers of *S. aureus* [7,8]; *Bacillus subtilis* (a Gram-positive bacterium) is known as the 'hay bacillus or grass bacillus', commonly found in soil, and more evidence suggests that *B. subtilis* is a normal gut commensal in humans [9]; *Pasteurella multocida*, a Gram-negative bacterium, causes a range of diseases in mammals and birds, including fowl cholera in poultry [10,11] and *Enterobacter cloacae*, a Gram-negative bacterium, is commonly found in infections among burn victims, immunocompromised patients, and patients with malignancies [12]. Development of new antibacterial agents is imperative. The increased prevalence of antibiotic-resistant bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control some bacterial diseases. Therefore, the search for new promising drugs and especially plant- drug derived may give a solution for the drug resistance crisis.

Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds. Lauraceae is one of the major families of plants, which is widespread in the tropical and subtropical regions, particularly in tropical America, Africa and Southeast Asia [13]. The Lauraceae or laurel family is known as "*Medang*" or "*Tejur*" by the Malays, and comprises a group of flowering plants included in the order Laurales; it consists of 55 genera and over 2,500 (perhaps as many as 4,000) species worldwide and about 16 genera and 213 species are found in Malaysia [14,15]. In Malaysia the species are largely montane. *Phoebe* species commonly grow most abundantly in Borneo and Peninsular Malaysia from central Perak to Malacca. *Phoebe* species are rich in alkaloidal constituents [16–18] and have been reported to contain the rare propaorphine-tryptamine dimers [19,20],

aporphines [19,21–25], proaporphine [19,25–27] and oxoaporphines [21,25,27] compounds. An interesting aporphine alkaloid named laurolitsine isolated from the stems of *P. formosana* (Hayata) has been used as a starting material to prepare bioactive phenanthrene alkaloids [28]. Some species of *Phoebe* also were reported in China, Indonesia, Indochina, Japan, The Philippines and the Malay Peninsula [15] to be useful to treat several diseases thanks to their antidiabetic, antibacterial and antifungal activities [24]. Aqueous extract of berries of *P. lanceolata* is an important remedy and has been used in the traditional medicine system in India for wounds and sores, whereas ethanolic extracts of *P. lanceolata* stem bark were evaluated for their antibacterial activity against five bacterial species: *Staphylococcus aureus* (along with ten hospital-derived strains), *Staphylococcus mutans*, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* with MIC range of 50–100 μg/mL [29].

Phoebe grandis is locally known in Malaysia as "*medang ketanah or tanah*" [30,31]. A proaporphine alkaloid which occurs in *P. grandis* is used as a precursor in the synthesis of aporphines and proaporphine-tryptamines alkaloids [25]. These alkaloids are known for their unique pharmacological activities, as demonstrated by liriodenine which shows antitumor, antibacterial, and antifungal activities [27]. Biological screening on the crude alkaloidal extract of the leaves of *Phoebe grandis* for antiplasmodial activity has shown positive results too: $IC_{50} < 8$ μg·mL^{-1} [32]. Although, most of the species of *Phoebe* are rich in alkaloidal contents, a few species are also reported as alkaloid-free [33]. We have launched a chemical investigation on the extract. Further investigation of the leaves has now led to the isolation of a known oxoaporphine alkaloid, lysicamine (**1**), and three proaporphine alkaloids, litsericinone (**2**), 8,9,11,12-tetrahydromecambrine (**3**) and hexahydro-mecambrine A (**4**) (Figure 1). Compounds **2** and **3** were isolated for the first time as natural compounds. Compound **4** has also been isolated from *Phoebe scortechinii* [34] and was previously synthesized by Nakasato [35].

Figure 1. Alkaloids **1**–**4** isolated from *Phoebe grandis* (Nees) Merr. (Lauraceae).



1 **2** **3** **4**

The objective of this research was to isolate and identify bioactive compounds from *Phoebe grandis* (Nees) Merr. (Lauraceae) with potential cytotoxicity against the MCF7 and HepG2 cell lines and test their antibacterial activities.

2. Results and Discussion

2.1. Compound characterization

Lysicamine (**1**) was obtained as a yellow amorphous solid. $[\alpha]_D^{29} = -25$ (c = 0.00008, $CHCl_3$). The mass spectrum showed the $[M+H]^+$ peak at *m/z* = 292.0963, which corresponds to a molecular formula of $C_{18}H_{13}NO_3$. (calcd. for $C_{18}H_{14}NO_3$, 292.0929). Other significant fragmentations observed were at *m/z* 277, which may be attributed to the loss of a CH_3 molecule, $[M-15]^+$. The UV spectrum showed absorption maxima at 236, 267, 360 and 396 nm, indicating the existence of a highly unsaturated oxoaporphine chromophore [36,37]. The IR spectrum showed a conjugated ketone peak at 1665 cm^{-1} [37,38]. The ^{1}H-NMR spectrum showed two distinct methoxyl peaks at δ 4.00 and δ 4.08, which were probably situated at C-1 and C-2. H-3 appeared as a singlet at δ 7.21. Two doublets (J = 5.2 Hz) typical of the H-4 and H-5 signals of an oxoaporphine were observed at δ 7.78 and δ 8.88, respectively. The H-5 proton was deshielded by the neighbouring N atom. A very downfield signal at δ 9.16 appeared as doublet of doublet with J_1 = 8.4 and J_2 = 0.7 belongs to H-11. In addition, a doublet of doublet peak was observed at δ 8.57 (1H, *dd*, J_1 = 7.9 Hz, J_2 = 1.4 Hz; H-8) which experienced a deshielding effect from the neighbouring C-7 carbonyl group. The peak appeared as doublet-triplet at δ 7.75 with J_1 = 8.52 Hz and J_2 = 1.64Hz was assigned for H-9 whereas H-10 resonated at δ 7.56 as doublet-triplet with J_1 = 8.52 Hz and J_2 = 1.64 Hz. The ^{13}C-NMR spectrum gave a total of eighteen carbons which validated the molecular formula of $C_{18}H_{13}NO_3$. Analysis of the ^{13}C-NMR spectrum gave nine quaternary carbons. Hence, compound **1** is an oxoaporphine alkaloid and in fact it was identified as lysicamine by the full agreement of the ^{1}H- and ^{13}C-NMR data of **1** with the literature values for that compound [39,40].

Litsericinone (**2**) was isolated as a yellow amorphous solid, $[\alpha]_D^{29} = -25$ (c = 0.00004, $CHCl_3$). This proaporphine alkaloid exhibited an $[M+H]^+$ peak in the LCMS-IT-TOF ESI (positive mode) mass spectrum at *m/z* 286.1421 which correlated to the molecular formula of $C_{17}H_{19}NO_3$ (calcd. for $C_{17}H_{20}NO_3$, 286.1432). The UV spectrum revealed three peaks at 300, 236 and 207 nm, which indicated the existence of a conjugated system [37]. The IR spectrum revealed a very significant carbonyl absorption at 1712.52 cm^{-1}. In addition, the presence of a methylenedioxyl group was proven by its characteristic absorption peaks at 1248.37 cm^{-1} and 934.90 cm^{-1}, which indicate asymmetric C-O-C stretching.

The ^{1}H-NMR spectrum showed one aromatic proton singlet at δ 6.51 which may be ascribed to H-3. H-6a resonated at δ 4.26 (*dd*, *J*, *J'* = 9.8, 6.9Hz). The methylenedioxyl protons appeared as a pair of doublets at δ 5.89 and δ 5.86 with J = 1.3 Hz. The aliphatic protons of ring D resonated as multiplets between δ 1.91 to 2.68, while H-7 appeared as multiplet at δ 2.73 and δ 1.87 as showed in HSQC spectrum. The above observations were reinforced by COSY experiment which showed correlations between vicinal protons; $H4_{ax}/H5_{ax}$, $H4_{eq}/H5_{eq}$, $H5_{ax}/H4_{ax}$, $H5_{eq}/H4_{eq}$, $H6a/H7_{eq}$, $H8_{eq}/H9_{eq}$, $H9_{eq}/H8_{eq}$,

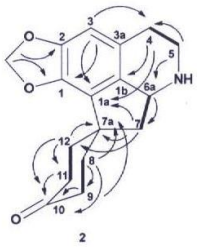
$H11_{ax}/H12_{ax}$ and $H12_{ax}/H11_{ax}$. Interestingly, H6a revealed correlation to $H7_{eq}$ suggesting the possibility that H6a is in an equatorial configuration.

The ^{13}C-NMR spectrum of litsericinone (**2**) showed the presence of seventeen carbon atoms, whereas the DEPT experiment displayed the presence of seven methylenes, a methylenedioxyl and two methine signals. The ^{13}C-NMR spectrum revealed the C-10 carbonyl peak at δ 211.12 and the quarternary carbon showed five signal peaks between δ 123.91 to 148.90. The characteristic proaporphine quarternary C-7a spirocarbon appeared at δ 46.14. The complete assignments of all protons and carbons (Table 1) were aided by the HSQC and HMBC experiments. The proximity of H6a and $H7_{eq}$ was confirmed by the HMBC correlation peaks between $H5_{eq}$/C6a and $H7_{eq}$/C6a. Figure 2 shows the HMBC and COSY correlation of litsericinone (**2**).

Table 1. ^{13}C-NMR (150 MHz), ^{1}H-NMR (600 MHz) and HMBC spectral data of litsericinone, (**2**) in $CDCl_3$ (δ in ppm, J in Hz).

Position	^{13}C (δ, $CDCl_3$)	Type	^{1}H (J, Hz)	HMBC (^{2}J, ^{3}J)
1	148.9	C_q	-	
1a	141.1	C_q	-	
1b	123.9	C_q	-	
2	141.1	C_q	-	
3	106.9	CH	6.51 s	C1, C1a, C2, C4
3a	126.7	C_q	-	
4	25.1	CH_2	2.87 m (ax) 2.75 m (eq)	C3, C1b, C1a C3, C1b, C1a
5	43.9	CH_2	3.55 m (ax) 3.16 m (eq)	C4, C6a, C1b C4, C6a
6a	56.8	CH	4.26 dd (J = 6.9 Hz, J = 9.8 Hz)	C5, C7
7	44.1	CH_2	2.73 m (ax) 1.87 m (eq)	C3, C3a, C1a C12, C7a, C6a
7a	46.2	C_q	-	-
8	38.5	CH_2	2.68 m (ax) 2.41 m (eq)	C10 C9, C7a, C10
9	36.2	CH_2	2.50 m (ax) 1.91 m (eq)	C8, C7a, C10 C11, C7a, C10
10	211.1	C=O	-	-
11	38.9	CH_2	2.46 m (ax) 2.45 m (eq)	C10 C12, C7a, C10
12	34.3	CH_2	2.10 m (ax) 2.00 m (eq)	C9, C7, C7a, C3a, C10 C9, C7a, C3a, C10
Methylenedioxy (O-CH_2-O)	100.9	CH_2	5.89 d (J = 1.3 Hz) 5.86 d (J = 1.3 Hz)	C1,C2 C1,C2

Figure 2. Key HMBC (→) and COSY (—) correlations of litsericinone (**2**).



8,9,11,12-Tetrahydromecambrine, (**3**) was isolated as a yellow amorphous solid, $[\alpha]_D^{29}$ = −25 (c = 0.00004, $CHCl_3$). The LCMS-IT-TOF mass spectrum of this proaporphine type of alkaloid showed a $[M+H]^+$ peak at *m/z* = 300.1596 which correlated to the molecular formula $C_{18}H_{21}NO_3$ (calcd. for $C_{18}H_{22}NO_3$, 300.1521). The UV spectrum showed an absorption peak at 203 nm. The IR spectrum showed a very significant carbonyl absorption peak at 1712.80 cm^{-1} due to C=O stretching vibrations. The presence of the methylenedioxyl group was proven by its characteristic absorption peaks at 1254.45 and 944.84 cm^{-1}, which indicate the asymmetric C-O-C stretching.

The ^{1}H-NMR spectrum displayed a pair of doublet peaks at δ 5.88 (d, *J* = 1.2 Hz) and 5.83 (d, *J* = 1.2 Hz) that correspond to the methylenedioxyl group at positions C-1 and C-2. A singlet peak appeared at δ 6.49 representing a proton attached to a benzene ring at the C-3 position. There is a strong singlet peak at δ 2.39 indicating a N-methyl group. The aliphatic protons appeared between δ 1.70 to 3.30. These peaks supported were by the COSY experiment that showed correlation peaks between $H5_{ax}/H4_{ax}$, $H4_{ax}/H5_{ax}$, $H6a/H7_{ax}$, $H7_{ax}/H6a$, $H6a/H7_{eq}$, $H7_{eq}/H6a$, $H11/H12_{ax}$, $H12_{ax}/H11$, $H11/H12_{eq}$ and $H12_{eq}/H11$. The ^{13}C-NMR spectrum of **3** showed the presence of eighteen carbons and the DEPT experiment showed the presence of seven methylene carbons (CH_2) and one methylenedioxyl group. The carbonyl group at C-10 position resonated at δ 211.65 and the N-methyl group at δ 43.47. The quaternary carbon at the C-7a position appeared at δ 46.04. The assignments of all carbons were established through the aid of the HSQC and HMBC experiments (Table 2). Figure 3 shows the HMBC and COSY correlation of the 8, 9, 11, 12-tetrahydromecambrine (**3**).

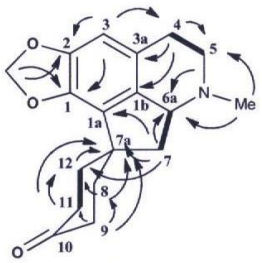
Table 2. ^{13}C-NMR (150 MHz), ^{1}H-NMR (600 MHz) and HMBC spectral data of 8,9,11,12-tetrahydromecambrine (**3**) in $CDCl_3$ (*δ* in ppm, *J* in Hz).

Position	^{13}C (δ, $CDCl_3$)	Type	^{1}H (*J*, Hz)	HMBC (^2J, ^3J)
1	140.7	C_q	-	-
1a	134.3	C_q	-	-
1b	124.5	C_q	-	-
2	148.2	C_q	-	-
3	106.5	CH	6.49 s	C1a, C1, C2,C4
3a	126.9	C_q	-	

Table 2. *Cont.*

Position	^{13}C (δ, $CDCl_3$)	Type	^{1}H (*J*,Hz)	HMBC (^{2}J,^{3}J)
4	27.4	CH_2	2.92 (m) ax	C1b, C5
			2.72 (m) eq	C1b, C1a, C3
5	55.0	CH_2	3.09 (m) ax	C1b, C4, NCH_3, C6a
			2.45 (m) eq	
6a	65.7	CH	3.30 br s	-
7	44.5	CH_2	2.59 (m) ax	C3a, C1a, C8, C7a, C6a
			1.75 (m) eq	C8, C12, C7a, C6a
7a	46.0	C_q	-	-
8	34.6	CH_2	2.14 (m) ax	C3a, C12, C7
			2.02 (m) eq	C3a, C12, C7a
9	39.0	CH_2	2.47 (m)	C8, C11, C7a
				C8, C11, C7a
10	211.7	C=O	-	-
11	38.6	CH_2	2.70 (m) ax	C12, C7a
			2.43 (m) eq	C12, C7a
12	36.5	CH_2	2.50 (m) ax	C8, C11, C7
			1.93 (m) eq	C3a, C8, C11, C7a
N-CH_3	43.5	CH_3	2.39 s	C5, C6a
Methlenedioxy	100.6	CH_2	5.88 *d* (*J* = 1.2)	C1, C2
(O-CH_2-O)			5.83 *d* (*J* = 1.2)	C1, C2

Figure 3. Key HMBC (→) and COSY (—) of 8,9,11,12-tetrahydromecambrine (**3**).



3

Hexahydromecambrine A (**4**) was isolated as an amorphous solid, $[\alpha]_D^{29}$ = +100 (c = 0.00003, $CHCl_3$). The mass spectrum showed a molecular ion peak at *m/z* = 302.9177 $[M+H]^+$, thus suggesting a molecular formula of $C_{18}H_{23}NO_3$ (calcd. for $C_{18}H_{24}NO_3$, 302.9167). The UV spectrum showed three peaks at 300, 245 and 265 nm, which indicate the existence of a conjugated system [37]. The IR spectrum showed a broad absorption band at 3391.78 cm^{-1}, indicating the presence of a hydroxyl group. The methylenedioxyl group absorbed at 1254.36 and 929.07 cm^{-1}. There was no carbonyl group present in the IR spectrum.

The ^{1}H-NMR for this alkaloid showed a pair of doublet peaks at δ 5.90 ppm (*d*, *J* = 1.20 Hz) and δ 5.86 ppm (*d*, *J* = 1.20 Hz) that correspond to the methylenedioxyl group at positions C-1 and C-2. A sharp singlet peak at 6.47 ppm belongs to the H-3 on the benzene ring. The aliphatic protons of ring D resonated as multiplets between δ 1.25 to 2.45. H-6a resonated as a multiplet at δ 3.26. A strong peak

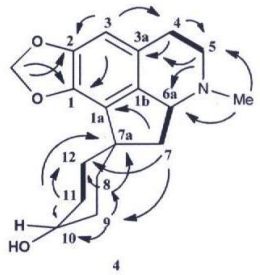
at δ 2.39 appeared as a sharp peak that was assigned for a N-methyl group. H-10, which is in proximity to a hydroxyl group, resonated further upfield at δ 4.00 as a broad multiplet, suggesting the possibility of an axial configuration. Interestingly, from the COSY spectra, H-10 was determined at be pseudo-axial by the correlations H-10 (δ 4.00) with H-8_{ax} (δ 2.03) and H-11 (δ 1.75). In the case of a pseudo-equatorial hydroxyl group, H-10 will resonate at around δ 4.26–4.39 [41].

The ^{13}C-NMR spectrum of **4** showed the presence of eighteen carbons, which is in agreement with the molecular formula $C_{18}H_{23}NO_3$. The DEPT spectrum showed the presence of one N-methyl, eight methylenes, three methines and six quaternary carbons. The methylenedioxyl group resonated at δ 100.5. The characteristic proaporphine quaternary spirocarbon peak C-7a appeared at δ 46.6. Apparently, C-10 resonated at δ 67.1, further strengthening the hypothesis of H-10 being axial. The complete assignments of all protons and carbons were aided by the HSQC and HMBC correlation and HMBC data were recorded in Table 3. Figure 4 showed HMBC and COSY correlations of hexahydromecambrine A. The structure of **4** resembled the structure of **3** except for the fact that the carbonyl group in **3** was reduced to a hydroxyl group in **4**.

Table 3. ^{13}C-NMR (150 MHz), ^{1}H-NMR (600 MHz) and HMBC spectral data of hexahydromecambrine A (**4**) in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	^{13}C (δ, $CDCl_3$)	Type	^{1}H (*J*, Hz)	HMBC (^{2}J, ^{3}J)
1	148.1	C_q	-	-
1a	129.0	C_q	-	-
1b	131.0	C_q	-	-
2	140.8	C_q	-	-
3	105.9	CH	6.46 (s)	C1, C2,C4
3a	124.0	C_q	-	-
4	27.3	CH_2	2.93 (m) ax	C5
			2.71 (m) eq	C3a
5	54.9	CH_2	3.11 (m) ax	C3a, C6a
			2.46 (m) eq	
6a	65.7	CH	3.26 (m)	-
7	44.3	CH_2	2.44 (m) ax	C1a
			1.58 (m) eq	C12, C9
7a	46.6	C_q	-	-
8	30.2	CH_2	2.03 (m) ax	C7a, C10
			1.54 (m) eq	C12, C7a, C10
9	31.7	CH_2	2.41 (m) ax	
			1.46 (m) eq	C8, C7a, C10
10	67.1	CH	4.00 br, m	
11	31.0	CH_2	1.75 (m)	C12, C7a, C10
12	29.7	CH_2	1.25 (m)	
N-CH_3	43.2	CH_3	2.39 (s)	C5, C6a
(OCH_2O)	100.5	CH_2	5.90 (*d*, *J* = 1.2)	C1, C2
			5.86 (*d*, *J* = 1.2)	C1, C2

Figure 4. Key HMBC (→) and COSY (—) of hexahydromecambrine A, (**4**).



4

2.2. Cell Culture and MTT Cytotoxicity Activity

A preliminary screening against cancer cells was performed using the MTT method [42] against MCF7 and HepG2 cell lines. The cytotoxicity of compounds **1–4** were assayed at various concentrations under continuous exposure for 24 h, are expressed in IC_{50} values (μg/mL), and are summarized in Table 4. Doxorubicin was used as a positive control in this study. The results showed compounds **1** and **2** exhibited cytotoxic activity against both cancer cell lines with IC_{50} values ranging from 14–60 μg/mL. Compounds **3** and **4** exhibited cytotoxic activity against the HepG2 cell line with IC_{50} values of 81 and 20 μg/mL, respectively. Compounds **3** and **4**, however, were not toxic towards the MCF7 cell line with IC_{50} values of more than 100 μg/mL.

Table 4. The IC_{50} values of compounds **1–4** and positive control on the MCF7 and HepG2 cell lines.

Compounds	IC_{50} (μg/mL) at 24 h	
	MCF7	HepG2
1	26	27
2	60	14
3	>100	81
4	>100	20
Doxorubicin	0.2	1.06

The results shown in Table 4 implied that the cytotoxic property were related to compounds with 7-oxo and methylenedioxy functions, which corresponds to the result previously described by Likhitwitayawuid *et al.* [43] that aporphine alkaloids containing a 1,2-methylenedioxy substituent appear to play a similar role in the oxoaporphine alkaloids, being required for the expression of cytotoxicity of the compounds. The aporphine alkaloid lysicamine (**1**) demonstrated moderate activity comparable to doxorubicin (positive control) against the MCF7 and HepG2 cell lines. It is important to notice that compounds **2–4** share the same basic skeleton with different substitution patterns. Interestingly, the proaporphine litsericinone (**2**), showed the highest cytotoxic potency against the HepG2 cell line (IC_{50} = 14 μg/mL) and MCF7 cell line (IC_{50} = 60 μg/mL) due to the presence of a methylenedioxy and a carbonyl group at the C10 position [43,44]. The enhanced cytotoxic activity of compound **4** against the HepG2 cell line implies the presence of a 10-hydroxyl in the molecule was generally results in better potency than a non-hydroxylated proaporphine. Similarly, the weak cytotoxic activity displayed by compound **3** compared to compound **2** may be due to the presence of

the *N*-methyl. These findings also suggested that the aromatic ring system, 7-oxo function, and methylenedioxy ring as well as the structural planarity have powerful effects on the cytotoxic activity against different cancer cell lines [45].

2.3. Antibacterial Activity

The preliminary screening results of antibacterial activity against five bacterial species are summarized in Table 5. Antibacterial activities were indicated by a clear zone of growth inhibition. Compounds **1**□**3** were evaluated for antibacterial activity using disc diffusion method. A standard antibiotic (streptomycin sulfate) was used as a positive control in the assay. Only these three compounds were tested only, as the amount of 4 available was insufficient to run the assays. The results indicated that lysicamine (**1**) exhibited a broader spectrum of activity against the microbes compared to the other two compounds—litsericinone (**2**) and 8,9,11,12-tetrahydromecambrine (**3**), which were found to be inactive.

Table 5. The inhibition zone diameter (in mm) of isolated compounds against selected bacteria.

Sample	Inhibition diameter (mm ± SD)				
	Staphylococcus epidermidis	***Staphylococcus aureus***	***Bacillus subtilis***	***Pasteurella multocida***	***Enterobacter cloacae***
	(Gram +ve)	**(Gram +ve)**	**(Gram +ve)**	**(Gram −ve)**	**(Gram −ve)**
1	12.00 ± 0.00	13.33 ± 0.57	15.50 ± 0.57	NI	NI
2	NI	NI	NI	NI	NI
3	NI	NI	NI	NI	NI
4	nt	nt	nt	nt	nt
Streptomycin sulfate [a]	20.00 ± 0.00	13.66 ± 0.57	21.00 ± 0.00	21.33 ± 1.15	NI

Note: (NI)—no inhibition observed, (nt)—not tested. Doses of the samples were 1 mg/mL per disc, Streptomycin sulfate 10 µg per disc. [a]—Positive control.

Lysicamine (**1**) displayed a strong antibacterial activity against *Staphylococcus aureus* with inhibition zones of 13.33 ± 0.57 mm, which are comparable with those of the positive control streptomycin sulfate. Moderate antibacterial activity was observed for compound **1** against *Staphylococcus epidermidis* and *Bacillus subtilis*, with inhibition zones of 12.00 ± 0.00 mm and 15.50 ± 0.57 mm, respectively. *Pasteurella multocida* and *Enterobacter cloacae* (both Gram-negative bacteria) were not susceptible to any of the isolated compounds. Generally, the results showed that compound **1** displayed strong activity against Gram-positive bacteria. The negative results obtained against the Gram-negative bacteria were not surprising, as in general, these bacteria are more resistant than Gram-positive ones [46–48]. These differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative cell wall is a multi-layered structure and quite complex [49,50], with an outer membrane consisting of a hydrophilic surface rich in lipopolysaccharide molecules [51]. In addition, the periplasmic space contains enzymes able to degrade any exogenous molecules and also prevent the entry of inhibitors, including antibiotic molecules [47,52]. Gram-positive bacteria do not possess this type of outer membrane and cell wall structure. Therefore, antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and produce a leakage of the cytoplasm and its coagulation [53]. The other two compounds

2 and **3** were not toxic towards any of the tested pathogenic bacteria since no appreciable zones of inhibition were observed. The present results regarding the antibacterial properties of the compounds from the leaves of *Phoebe grandis* (Nees) Merr. indicate that compounds from the plants of this genus could be used against the most common Gram-positive pathogen, and that their potential activity is probably due to their ability to complex with extracellular and soluble proteins and bacterial cell walls.

3. Experimental

3.1. General

Optical rotations were obtained on a JASCO P-1020 polarimeter. UV spectra were measured using Shimadzu UV-1650 PC Ultraviolet-Visible Spectrophotometer. The solvent used was methanol (CH_3OH) while the wavelength in which the spectrum was recorded is 200–400 nm. IR spectra were recorded in $CHCl_3$ on a Perkin Elmer Spectrum 2000-FTIR Spectrometer. NMR spectra were recorded in deuterated chloroform ($CDCl_3$) on Bruker AVN instruments (400 and 600 MHz for ^1H and 100 and 150 MHz for ^{13}C, respectively). Chemical shifts reported in ppm on the δ scale and the coupling constants are given in Hz. Spectra signals were calibrated using TMS. Mass spectra were obtained using a Shimadzu LCMS-IT-TOF instrument. The solvent used was chloroform ($CHCl_3$). Silica gel 60F, 70–230 mesh ASTM (Merck 7734); silica gel 60F, 230–400 mesh ASTM (Merck 9385); silica Gel $60GF_{254}$, (Merck 1.07730.1000) were used for chromatographic separations. Thin Layer Chromatography analysis was performed by using aluminium supported silica gel $60F_{254}$ TLC sheets (Merck 1.05554.0001) or glass supported silica gel $60F_{254}$ TLC plates (Merck 1.05715.0001). Mayer's and Dragendorff's reagents were used for alkaloid screening to identify the presence of the alkaloids and alkaloid spotting (TLC).

3.2. Plant Material

Leaves of *Phoebe grandis* (Nees) Merr. (Lauraceae) were collected on 19 February 2008 from the Bukit Serting Forest Reserve, Negeri Sembilan, Malaysia and the voucher specimen (KL 5540) was deposited in the Herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia and in the Herbarium of the Forest Research Institute, Kepong, Malaysia. The plant (KL 5540) was identified by Mr Teo Leong Eng, a botanist from the Department of Chemistry Herbarium, University of Malaya, Kuala Lumpur, Malaysia.

3.3. Extraction and Isolation of the Alkaloids

Dried and milled leaves of *Phoebe grandis* (Nees) Merr. (4.0 kg) were first defatted with hexane (7 L) for 3 days at room temperature, then filtered. After that they were moistened with 15% of NH_4OH, and exhaustively extracted with CH_2CI_2 using a Soxhlet extractor for about 18 h. The CH_2CI_2 extract was reduced to 500 mL followed by acidic extraction using 5% HCI until Mayer's test was negative. The combined extracts were then made alkaline with concentrated ammonia solution to pH 10–11 and re-extracted with CH_2CI_2. The CH_2CI_2 fractions were washed with distilled H_2O and dried over anhydrous sodium sulphate. The dichloromethane extract was evaporated to dryness under reduced pressure to give the crude extract.

The plant residue was extracted with methanol and the methanol was evaporated to dryness and then acidified by the addition of 5% hydrochloric acid solution and left to stand overnight. The acid solution was then filtered and made alkaline with 10% ammonia solution and reextracted with dichloromethane. The dark residue obtained after washing, drying and evaporating to dryness was added to the crude alkaloid obtained from the dichloromethane extracts to yield 13.28 g of crude alkaloids. This crude alkaloid mixture was subjected to column chromatography over silica gel using various ratios of CH_2Cl_2 and MeOH (100:0, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10, 88:12, 80:20, 70:30, 60:40, 50:50) and finally with pure 100% MeOH. The collected fractions were grouped into a series of fractions, monitored with TLC. Each series were then treated separately to isolate and purify its alkaloid by extensive column chromatography followed by preparative TLC.

3.4. Cell Culture and MTT Cytotoxicity Assay

The MCF-7 and HepG2 cells that were used in this study were obtained from the American Type Cell Collection (ATCC). The cells were cultured using RPMI 1640 culturing media (PAA, Leverkusen, Germany). Cells were trypsinized and counted using hemocytometer and plated in a microtiter plate of 96-wells. After an overnight incubation to allow cell attachment, the medium was changed and 0.2 mL of new supplemented medium were added into each well. Cells were then treated with the different drug concentrations and incubated at 37 °C, 5% CO_2 for 24 h. Each concentration of the samples was assayed in triplicate. The colorimetric assay were performed at an absorbance of 570 nm. Results were expressed as a percentage of control giving a certain percentage of cell viability after 24 h exposure to the test agent. The potency of cell growth inhibition for test agent was expressed as an IC_{50} value.

3.5. Bacterial Cultures and Disc Diffusion Assay

The *in vitro* antibacterial activity of the alkaloids was evaluated against five pathogenic microorganisms, including three Gram positive bacteria: *Staphylococcus epidermidis* (a clinically isolated strain), *Staphylococcus aureus* (S1434), *Bacillus subtilis* (B145) and two Gram negative bacteria: *Pasteurella multocida* (a clinically isolated strain) and *Enterobacter cloacae* (a clinically isolated strain). All the strains were stored in the appropriate medium before use.

Antibacterial activity of the alkaloids **1–3** was determined by the disc diffusion method [54] with slight modifications in terms of sample concentration, volume of sample loaded and use of paper discs. The bacteria were cultured at 37 °C for overnight in nutrient broth and potato dextrose broth, respectively. The concentrations of the cultures were adjusted turbidometrically at a wavelength of 600 nm which gave 10^5–10^6 colony forming units (CFU) per mL. The compounds to be tested were dissolved in dimethyl sulphoxide (DMSO) at concentration of 1 mg/mL. About 10 µL of each sample solution was loaded on Whatman No. 1 filter paper disc (Ø6 mm). The disc was placed on the surface of the agar plate (nutrient agar or potato dextrose agar) previously inoculated with bacteria. The agar plates were then inverted and incubated for 24 h at 37 °C. The antimicrobial activity was recorded by measuring the zone of inhibition (IZ) in mm around each disc, against the test organisms. The experiments were repeated in triplicate and the results were expressed as average values. The antibiotic streptomycin sulfate (10 µg/disc), was used as positive control and DMSO as negative control in the

assay. Compound **4** was not evaluated due to the small amount of compound available, not enough for antibacterial screening.

4. Conclusions

In general, alkaloids from the leaves of *Phoebe grandis* (Nees) Mer. (Lauraceae) showed a good *in vitro* cytotoxic activity against MCF7 and HepG2 and antibacterial activity against the selected bacteria. Lysicamine (**1**) showed the most promising activity compared to other compounds in the study. This study also suggest that the presence of a methylenedioxy group, unsaturated carbonyl group, hydrogen bond donor (OH group) and dimethoxy substituent in the compounds contributes to the multiple biological activities displayed. Therefore, structure-active relationships (SAR) can further investigated to determine the potency of related compound. In a nutshell, this study can be as a basis form for selection of plant species for further investigation in drug discovery for potential new natural bioactive compounds.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/18/8/8994/s1.

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Conflict of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available from the authors.

Article

In Vitro Antiplasmodial Activity of Aporphine Alkaloids Isolated from leaves of *Phoebe tavoyana* (Meissn.) H.k.F.

Hanita Omar[a,b,*], Aty Widyawaruyanti[c], Mohd Azlan Nafiah[d], Tiah Rachmatiah[e,f], Mehran Fadaeinasab[a], Ainnul Hamidah Syahadah Azizan[a], Najihah Mohd. Hashim[g], A. Hamid A. Hadi[a], Hapipah Mohd Ali[a].

[a] Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; E-mails: ainnul_azizan@yahoo.com (A.H.S.A.); ahamid@um.edu.my (A-H.A.H).

[b] Centre for Foundation Studies in Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

[c] Faculty of Pharmacy, Airlangga University, Jalan Dharmawangsa Dalam, Surabaya 60286, Indonesia.

[d] Chemistry Department, Faculty of Science and Mathematic, University of Pendidikan Sultan Idris, Tg. Malim, Perak, Malaysia; Emails: azlan@upsi.edu.my (M.A.N).

[e] Department of Chemistry, Faculty of Science and Mathematic, University of Indonesia, Depok, Indonesia; E-mail: tiahrachmatiah@yahoo.com (T.R.)

[f] Department of Pharmacy, Faculty of Science and Mathematic, National Institute of Science and Technology, Jakarta, Indonesia.

[g] Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; E-Mails: najihahmh@gmail.com (N.M.H.).

* Author to whom correspondence should be addressed. E-Mail: E-Mails: hanita74@um.edu.my (H.O.); Tel: 603-7967****, Fax: 603-79674193.

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Abstract: Two new aporphine named; tavoyanine A (**1**) and tavoyanine B (**2**) along with four known aporphines; laetanine (**3**), roemerine (**4**), laurolitsine (**5**) and boldine (**6**); and one morphinandienone type, sebiferine (**7**) were isolated from the leaves of *Phoebe tavoyana* (Meissn.) H.k.F. (Lauraceae). The isolation was achieved by chromatographic techniques and the structural elucidation was performed via spectral methods notably, UV, IR, HRESIMS and 1D- and 2D-NMR (COSY, HMQC, HMBC and NOE-DIFF). This paper also reports the antiplasmodial activity of roemerine (**4**), laurolitsine (**5**), boldine (**6**) and sebiferine (**7**). The results showed that (**4**), (**5**), (**6**) and (**7**) have shown potent inhibitory activity against the growth of *Plasmodium falciparum* 3D7 clone, with IC_{50} 0.89, 1.49, 1.65, and 2.76µg/mL respectively.

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Methanol Extract of *Actinodaphne sesquipedalis* (Lauraceae) enhance gastric defence against ethanol-induced gastric ulcer in mice

Hanita Omar [1,2], Shahram Golbabapour [3], Pouya Hassandarvish [3], Maryam Hajrezaie [3], Ainnul Hamidah Syahadah Azizan [1], Noraziah Nordin [1,3], Mehran Fadaei Nasab [1], Nazia Abdul Majid [4], Mahmood Ameen Abdulla [5], Berna Elya [6], Najihah Mohd. Hashim [3], Hapipah Mohd. Ali [1].

[1]*Chemistry Department, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia.*
Email: hanita74@um.edu.my, mehranfadaie_n@yahoo.com, ahamid@um.edu.my
[2]*Centre for Foundation Studies in Science, University of Malaya, 50603, Kuala Lumpur, Malaysia.*
Email: hanita74@um.edu.my
[3]*Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.*
Email: aziereality@yahoo.com
[4]*Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.*
[5]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.*
[6]*Faculty of Pharmacy, University of Indonesia, Kampus UI Depok 16424, Indonesia*

A b s t r a c t

Actinodaphne sesquipedalis Hook. F. Var. Glabra (Kochummen), belongs to the Lauraceae family is native to Myanmar, Thailand, Peninsular Malaysia, and Borneo Island. *Actinodaphne sesquipedalis* called as " *Medang payung*" by the Malay people which alluding to the arrangement of leaves in starshaped whorls [1]. We studied the anti-ulcer activity and acute toxicity of *Actinodaphne sesquipedalis* leaves, bark and fruits of methanolic extract for the first time.

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Introduction

Peptic ulcer anatomically can be divided into two categories; gastric ulcer and duodenal ulcer. Gastric ulcer is an open sore, erosion or lesion occurs at the lining of the stomach or duodenum, where hydrochloric acid and pepsin are present. Pathologically, disruption of the gastric mucosal defensive barrier leads to gastric ulcer [2]. In fact, gastric ulcers could occurred, provided imbalance between the protective factors such as mucus and bicarbonate, and destructive factors [3,4]. Destructive factors are varied, from natural causes (gastric cancer), infections (*Helicobacter pylori*), lifestyle (drugs use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDS) which stimulate gastric acid and pepsin secretion, alcohol consumption, stressful lifestyle, cigarette

APPENDIX A

Cytotoxic activity of crude extracts by using MTT assay

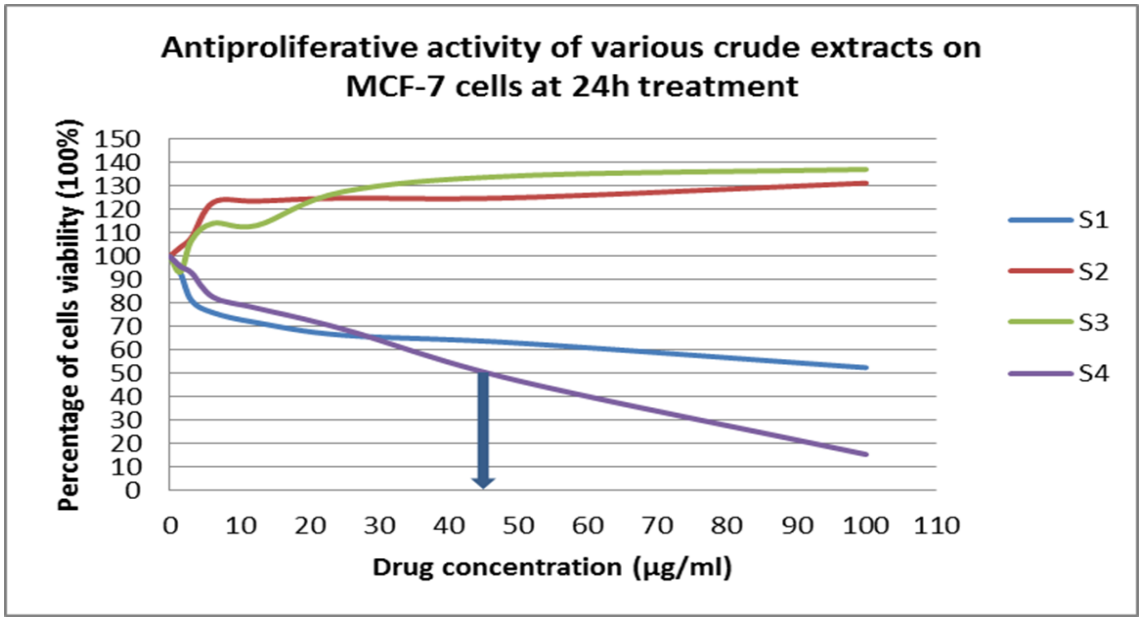


Figure 1: Percentage viability of MCF-7 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. grandis* measured after 24 hours using MTT assay.
IC_{50} value of S4 = 45.00 µg/mL.
S1, S2, S3 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: CH_2Cl_2 extract from fruits of *A. sesquipedalis* (S1), ethyl acetate extract from from fruits of *A. sesquipedalis* (S2), MeOH extract (acid-base) from bark of *A. sesquipedalis* (S3) and CH_2Cl_2 extract (acid-base) from bark of *P. grandis* (S4).

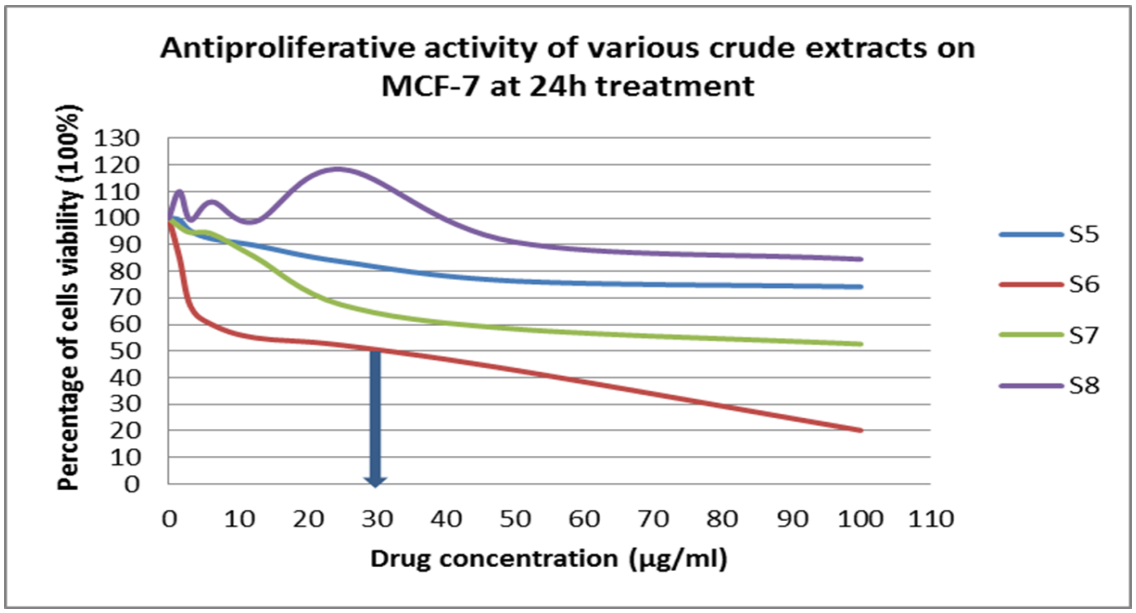


Figure 2: Percentage viability of MCF-7 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay.
IC_{50} value of S6 = 30.00 µg/mL.
S5, S7, S8 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: MeOH extract from leaves of *A. sesquipedalis* (S5), CH_2Cl_2 extract from leaves of *A. sesquipedalis* (S6), Hexane extract from fruits of *A. sesquipedalis* (S7) and CH_2Cl_2 extract from bark of *P. tavoyana* (S8).

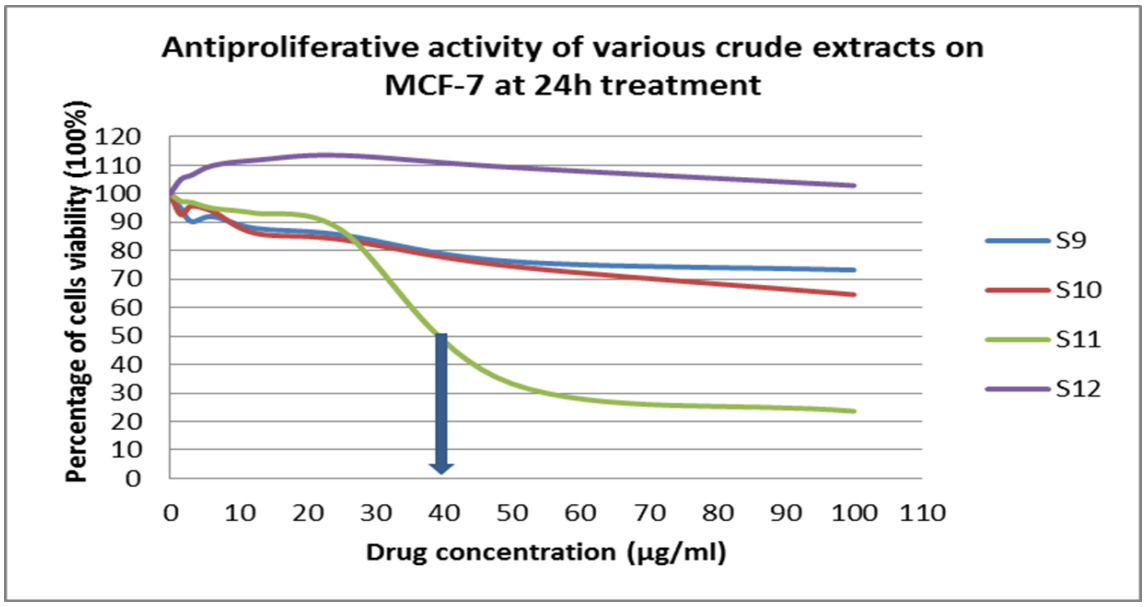


Figure 3: Percentage viability of MCF-7 cells treated with different concentration of extracts of *P. tavoyana* and *A. sesquipedalis* measured after 24 hours using MTT assay.
IC_{50} value of S11 = 40.00 µg/mL.
S9, S10, S12 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: Hexane extract from bark of *P. tavoyana* (S9), hexane extract from leaves of *A. sesquipedalis* (S10), CH_2Cl_2 (acid-base) extract from bark of *P. tavoyana* (S11) and MeOH extract from fruits of *A. sesquipedalis* (S12).

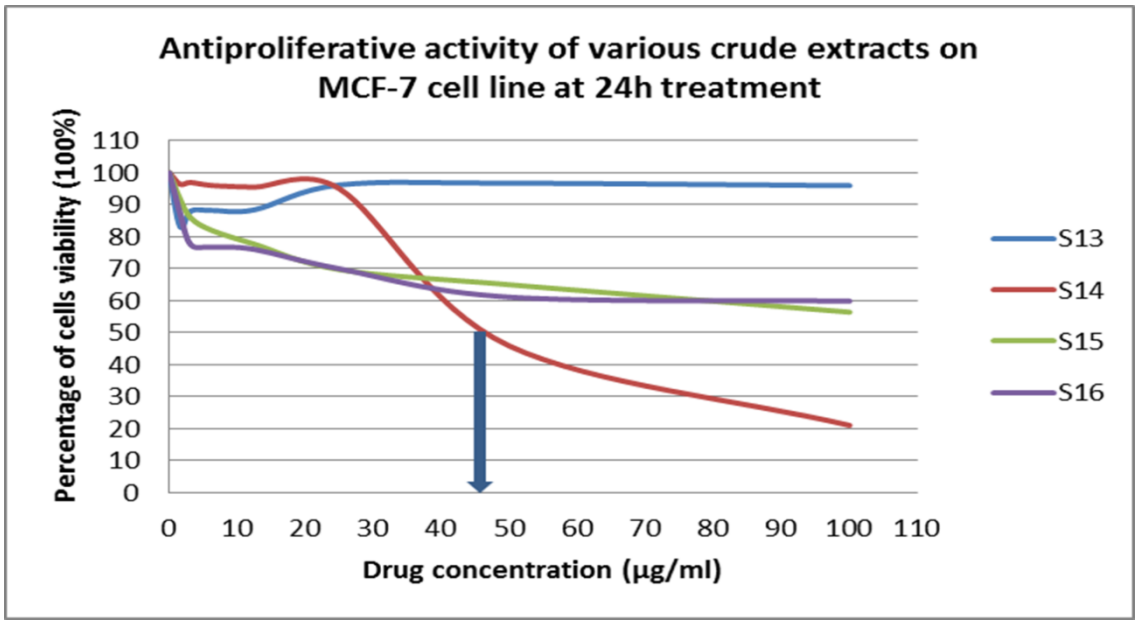


Figure 4: Percentage viability of MCF-7 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay.
IC_{50} value of S14 = 48.00 µg/mL.
S13, S15, S16 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: MeOH extract from bark of *A. sesquipedalis* (S13), $CHCl_3$ (acid-base) extract from bark of *P. tavoyana* (S14), hexane extract from leaves of *P. tavoyana* (S15) and CH_2Cl_2 extract from leaves of *P. tavoyana* (S16).

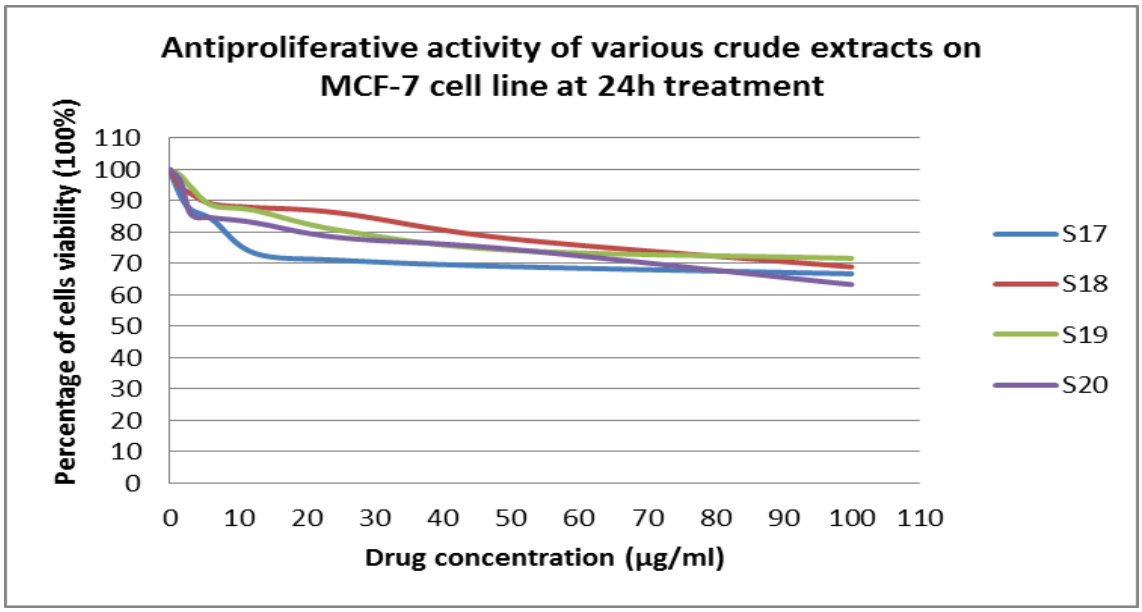


Figure 5: Percentage viability of MCF-7 cells treated with different concentration of extracts of *P. grandis* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.

Note: CH_2Cl_2 (acid-base) extract from leaves of *P. grandis* (S17), CH_2Cl_2 extract from leaves of *P. grandis* (S18), MeOH extract from leaves of *P. grandis* (S19) and hexane extract from leaves *of P. grandis* (S20).

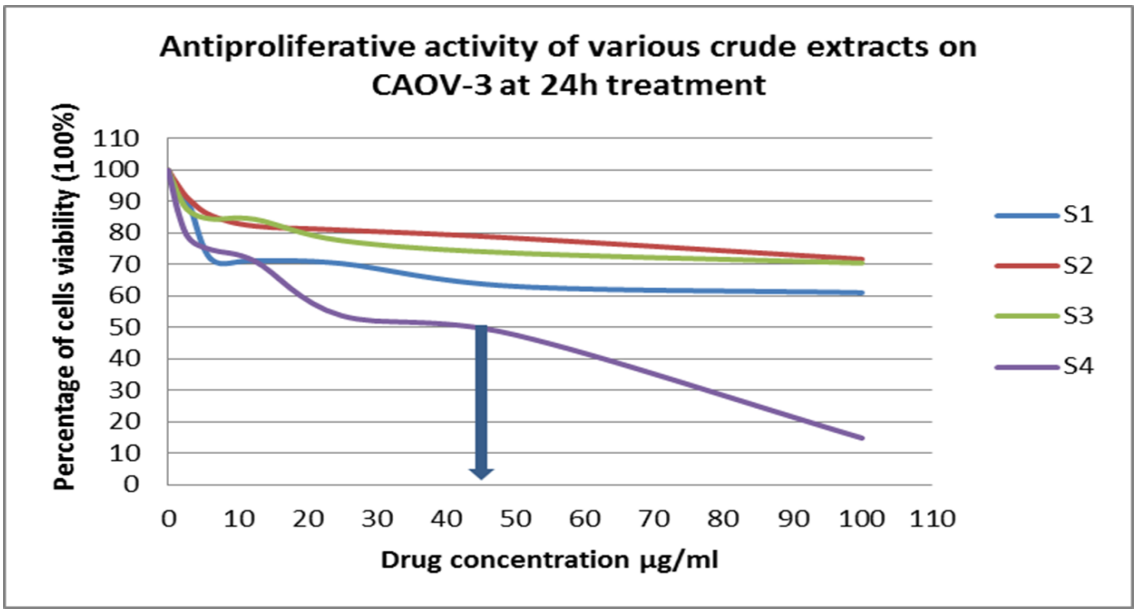


Figure 6: Percentage viability of Caov-3 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. grandis* measured after 24 hours using MTT assay.
IC_{50} value of S4 = 45.00 µg/mL.
S1, S2, S3 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: CH_2Cl_2 extract from fruits of *A. sesquipedalis* (S1), ethyl acetate extract from from fruits of *A. sesquipedalis* (S2), MeOH extract (acid-base) from bark of *A. sesquipedalis* (S3) and CH_2Cl_2 extract (acid-base) from bark of *P. grandis* (S4).

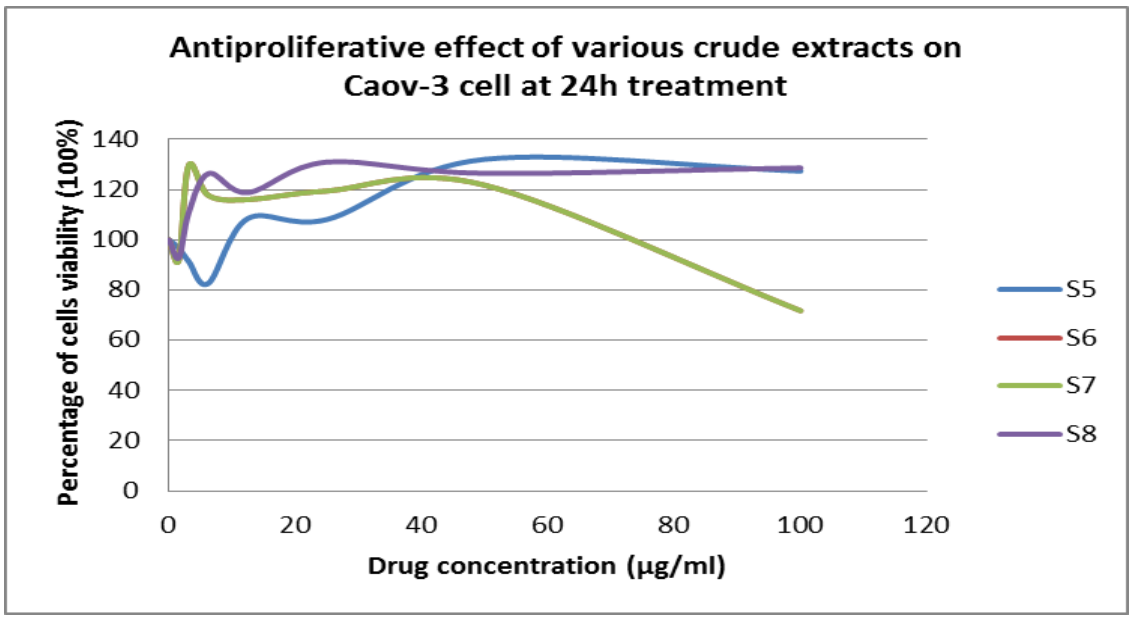


Figure 7: Percentage viability of Caov-3 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: MeOH extract from leaves of *A. sesquipedalis* (S5), CH_2Cl_2 extract from leaves of *A. sesquipedalis* (S6), Hexane extract from fruits of *A. sesquipedalis* (S7) and CH_2Cl_2 extract from bark of *P. tavoyana* (S8).

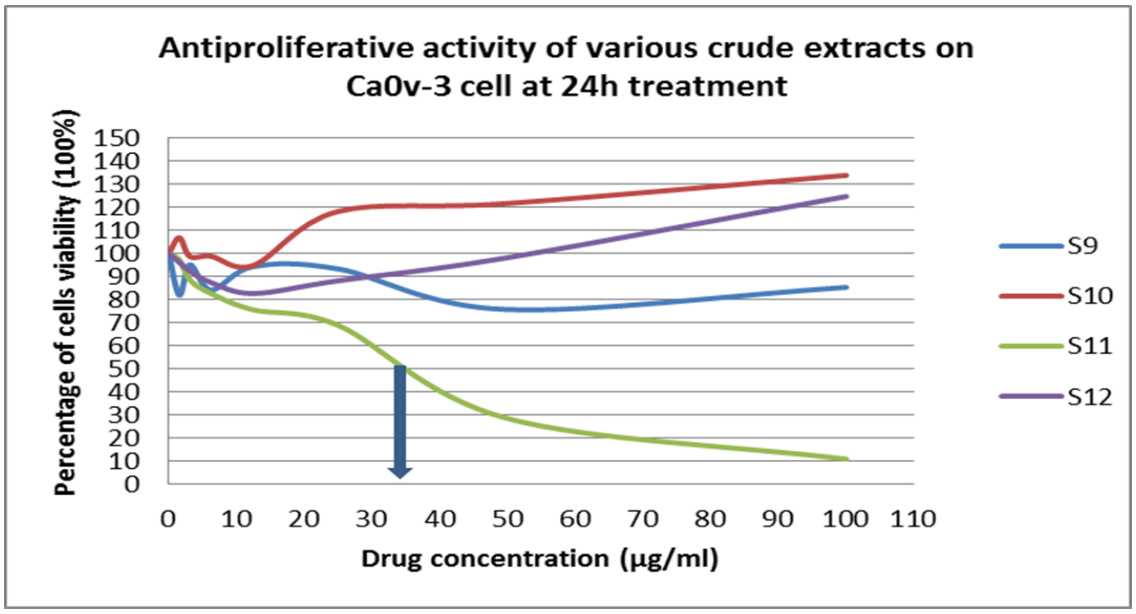


Figure 8: Percentage viability of Caov-3 cells treated with different concentration of extracts of *P. tavoyana* and *A. sesquipedalis* measured after 24 hours using MTT assay.
IC_{50} value of S11 = 35.00 µg/mL.
S9, S10, S12 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: Hexane extract from bark of *P. tavoyana* (S9), hexane extract from leaves of *A. sesquipedalis* (S10), CH_2Cl_2 (acid-base) extract from bark of *P. tavoyana* (S11) and MeOH extract from fruits of *A. sesquipedalis* (S12).

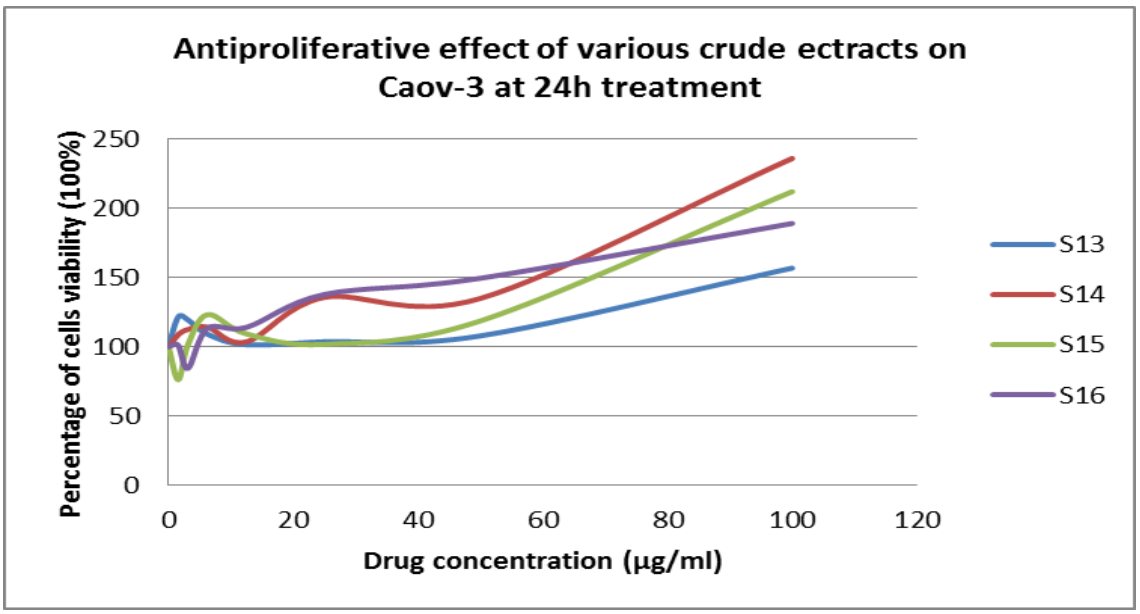


Figure 9: Percentage viability of Caov-3 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: MeOH extract from bark of *A. sesquipedalis* (S13), $CHCl_3$ (acid-base) extract from bark of *P. tavoyana* (S14), hexane extract from leaves of *P. tavoyana* (S15) and CH_2Cl_2 extract from leaves of *P. tavoyana* (S16).

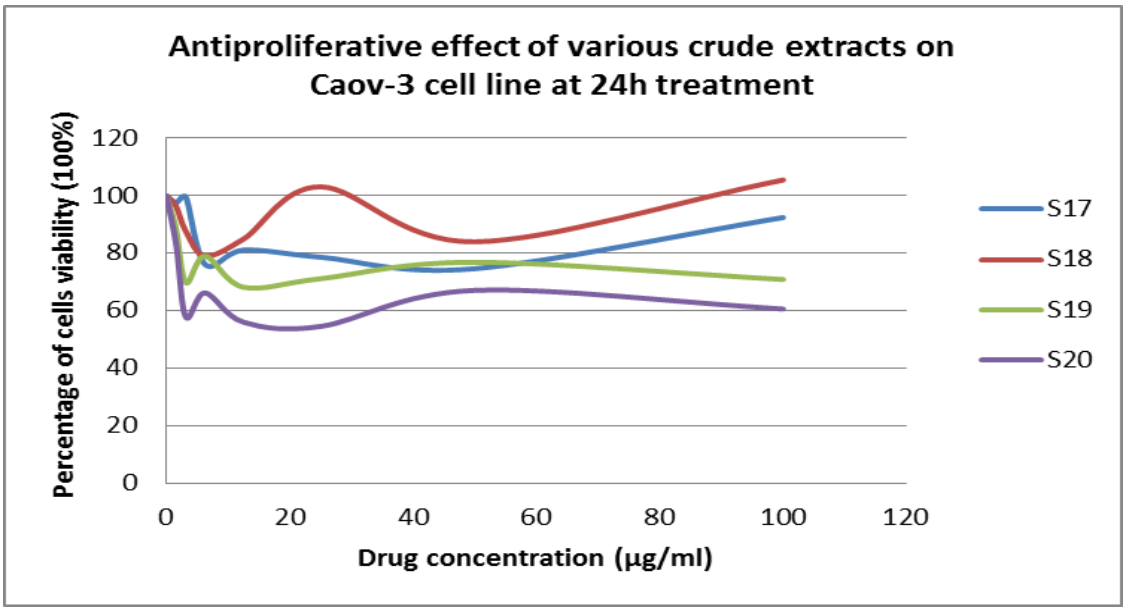


Figure 10: Percentage viability of Caov-3 cells treated with different concentration of extracts of *P. grandis* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.

Note: CH_2Cl_2 (acid-base) extract from leaves of *P. grandis* (S17), CH_2Cl_2 extract from leaves of *P. grandis* (S18), MeOH extract from leaves of *P. grandis* (S19) and hexane extract from leaves *of P. grandis* (S20).

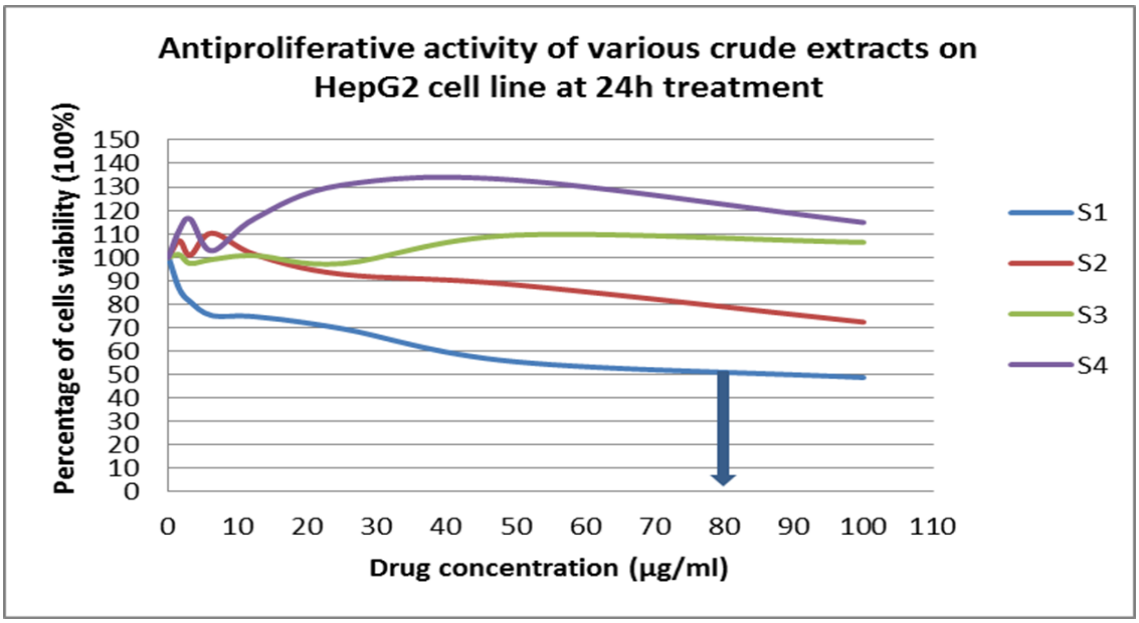


Figure 11: Percentage viability of HepG2 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. grandis* measured after 24 hours using MTT assay.
IC_{50} value of S1 = 80.00 µg/mL.
S2, S3, S4 = No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: CH_2Cl_2 extract from fruits of *A. sesquipedalis* (S1), ethyl acetate extract from from fruits of *A. sesquipedalis* (S2), MeOH extract (acid-base) from bark of *A. sesquipedalis* (S3) and CH_2Cl_2 extract (acid-base) from bark of *P. grandis* (S4).

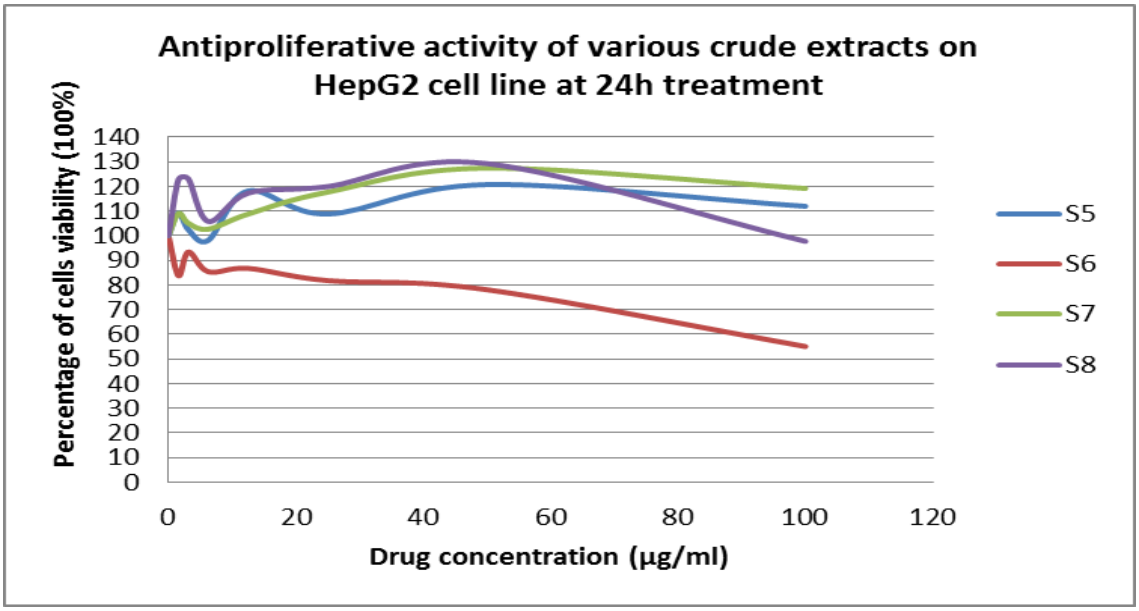


Figure 12: Percentage viability of HepG2 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.
Note: MeOH extract from leaves of *A. sesquipedalis* (S5), CH_2Cl_2 extract from leaves of *A. sesquipedalis* (S6), Hexane extract from fruits of *A. sesquipedalis* (S7) and CH_2Cl_2 extract from bark of *P. tavoyana* (S8).

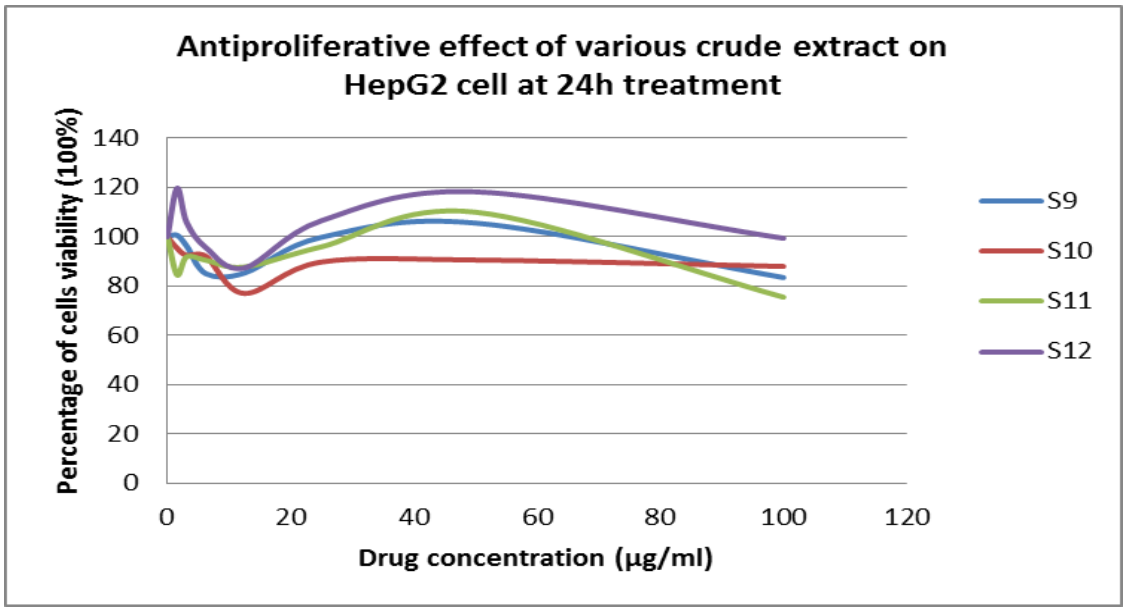


Figure 13: Percentage viability of HepG2 cells treated with different concentration of extracts of *P. tavoyana* and *A. sesquipedalis* measured after 24 hours using MTT assay. **No IC_{50} value or IC_{50} values are > 100.00 µg/mL.**

Note: Hexane extract from bark of *P. tavoyana* (S9), hexane extract from leaves of *A. sesquipedalis* (S10), CH_2Cl_2 (acid-base) extract from bark of *P. tavoyana* (S11) and MeOH extract from fruits of *A. sesquipedalis* (S12).

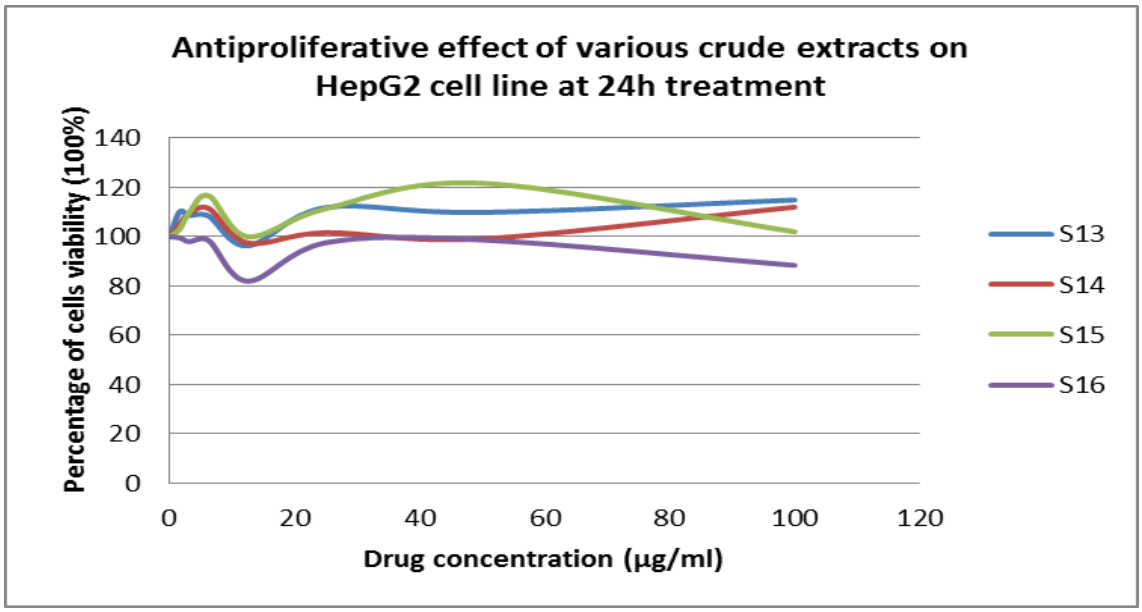


Figure 14: Percentage viability of HepG2 cells treated with different concentration of extracts of *A. sesquipedalis* and *P. tavoyana* measured after 24 hours using MTT assay. **No IC_{50} value or IC_{50} values are > 100.00 µg/mL.**

Note: MeOH extract from bark of *A. sesquipedalis* (S13), $CHCl_3$ (acid-base) extract from bark of *P. tavoyana* (S14), hexane extract from leaves of *P. tavoyana* (S15) and CH_2Cl_2 extract from leaves of *P. tavoyana* (S16).

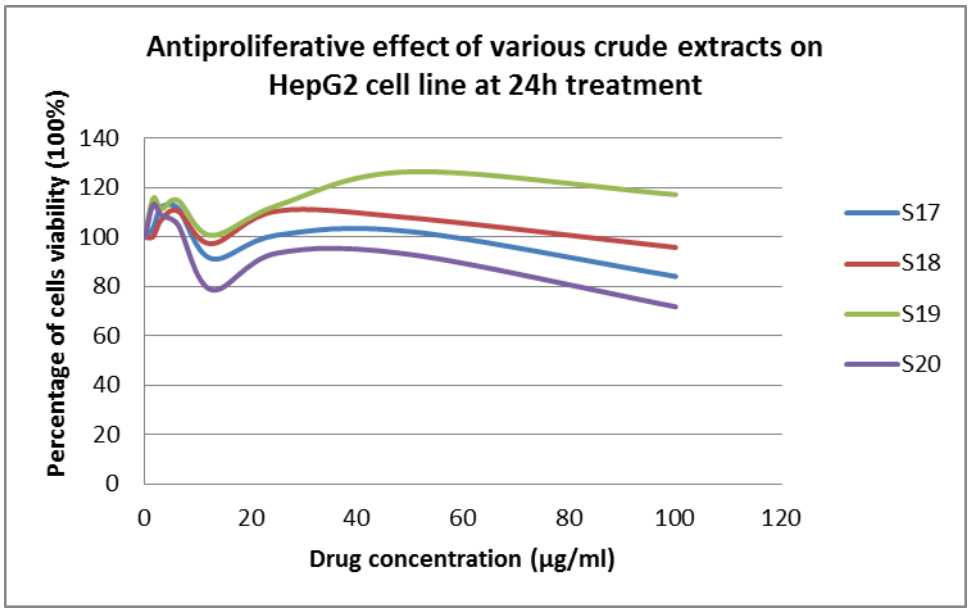


Figure 15: Percentage viability of HepG2 cells treated with different concentration of extracts of *P. grandis* measured after 24 hours using MTT assay.
No IC_{50} value or IC_{50} values are > 100.00 µg/mL.

Note: CH_2Cl_2 (acid-base) extract from leaves of *P. grandis* (S17), CH_2Cl_2 extract from leaves of *P. grandis* (S18), MeOH extract from leaves of *P. grandis* (S19) and hexane extract from leaves *of P. grandis* (S20).

APPENDIX B

FRAP values of extract of *P. grandis, P. tavoyana* and *A. sesquipedalis.*

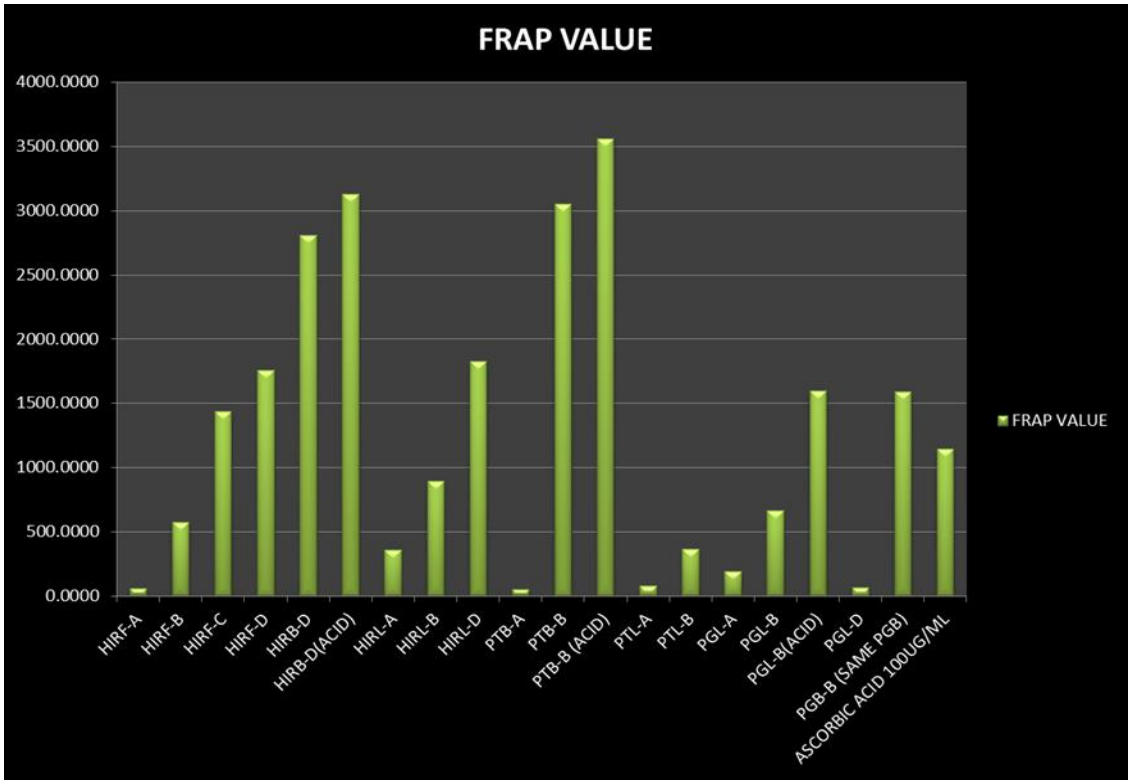


Figure 1: Graph of the FRAP values of extract of *P. grandis, P. tavoyana* and *A. sesquipedalis* measured after 4 minutes incubation.

Note: HIRF-A = Hexane extract of fruits of *A. sesquipedalis*.

HIRF-B = CH_2Cl_2 extract of fruits of *A. sesquipedalis*.

HIRF-C = Ethyl acetate extract of fruits of *A. sesquipedalis.*

HIRF-D = MeOH extract of fruits of *A. sesquipedalis.*

HIRB-D = MeOH extract of bark of *A. sesquipedalis.*

HIRB-D (ACID) = MeOH (acid-base) extract of bark of *A. sesquipedalis.*

HIRL-A = Hexane extract of leaves of *A. sesquipedalis*.

HIRL-B = CH_2Cl_2 extract of leaves of *A. sesquipedalis*.

HIRL-D = MeOH extract of leaves of *A. sesquipedalis*.

PTB-A = Hexane extract of bark *of P. tavoyana.*

PTB-B = CH_2Cl_2 extract of bark *of P. tavoyana.*

PTB-B (ACID) = CH_2Cl_2 (acid-base) extract of bark *of P. tavoyana.*

PTL-A = Hexane extract of leaves *of P. tavoyana.*

PTL-B = CH_2Cl_2 extract of leaves *of P. tavoyana.*

PGL-A = Hexane extract of leaves of *P. grandis*

PGL-B = CH_2Cl_2 extract of leaves of *P. grandis.*

PGL-B (ACID) = CH_2Cl_2 (acid-base) extract of leaves of *P. grandis.*

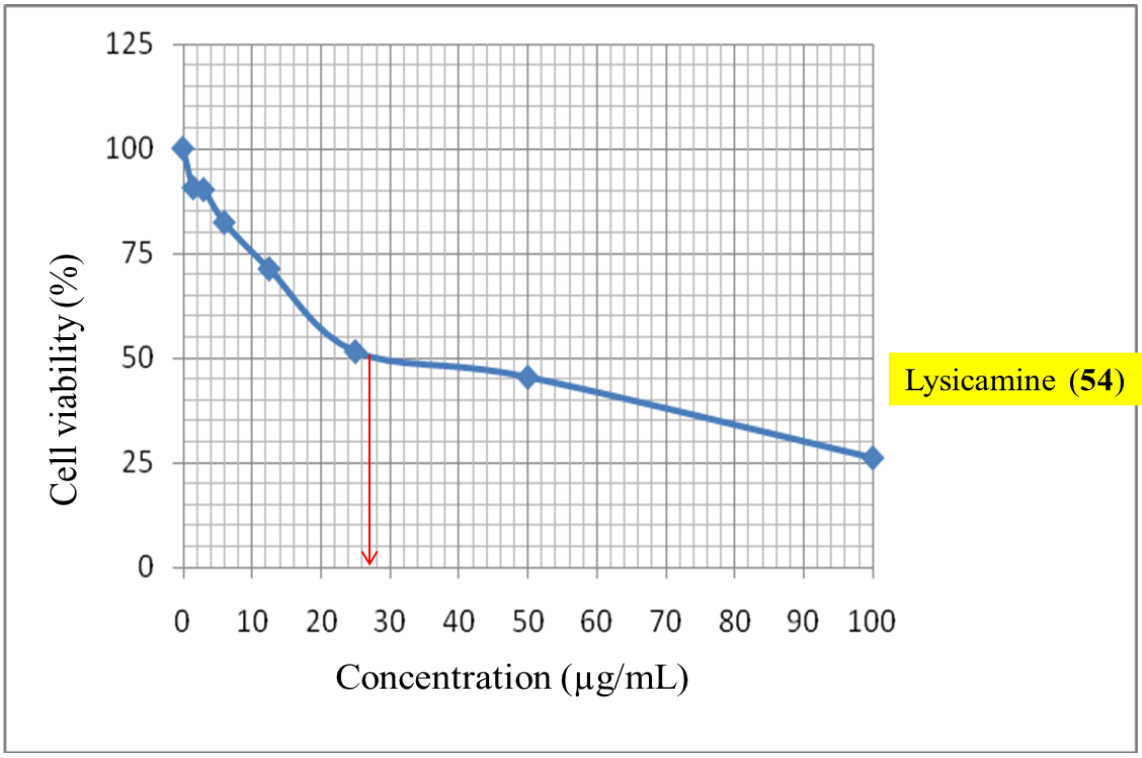
PGL-D = MeOH extract of leaves of *P. grandis.*

PGB-B (SAME PGB) = CH_2Cl_2 (acid-base) of bark of *P. grandis.*

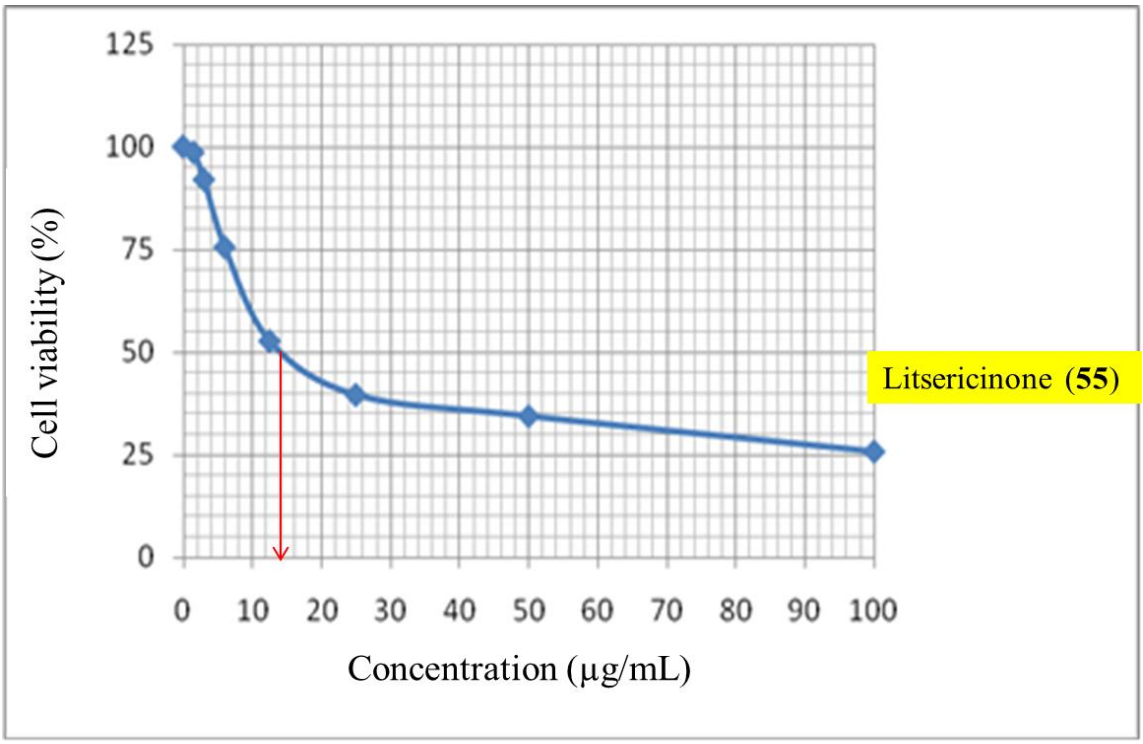
APPENDIX C

Cytotoxic activity of isolated compounds

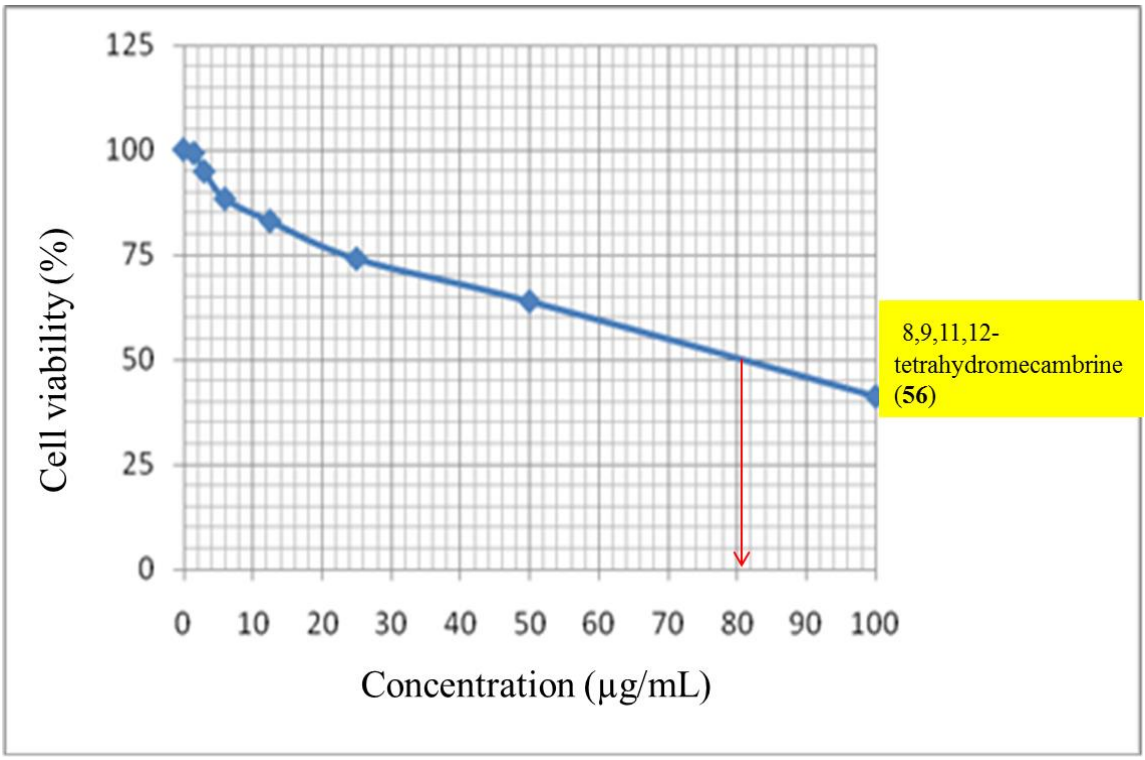
Cytotoxic activity of isolated compounds by using MTT assay against HepG2.



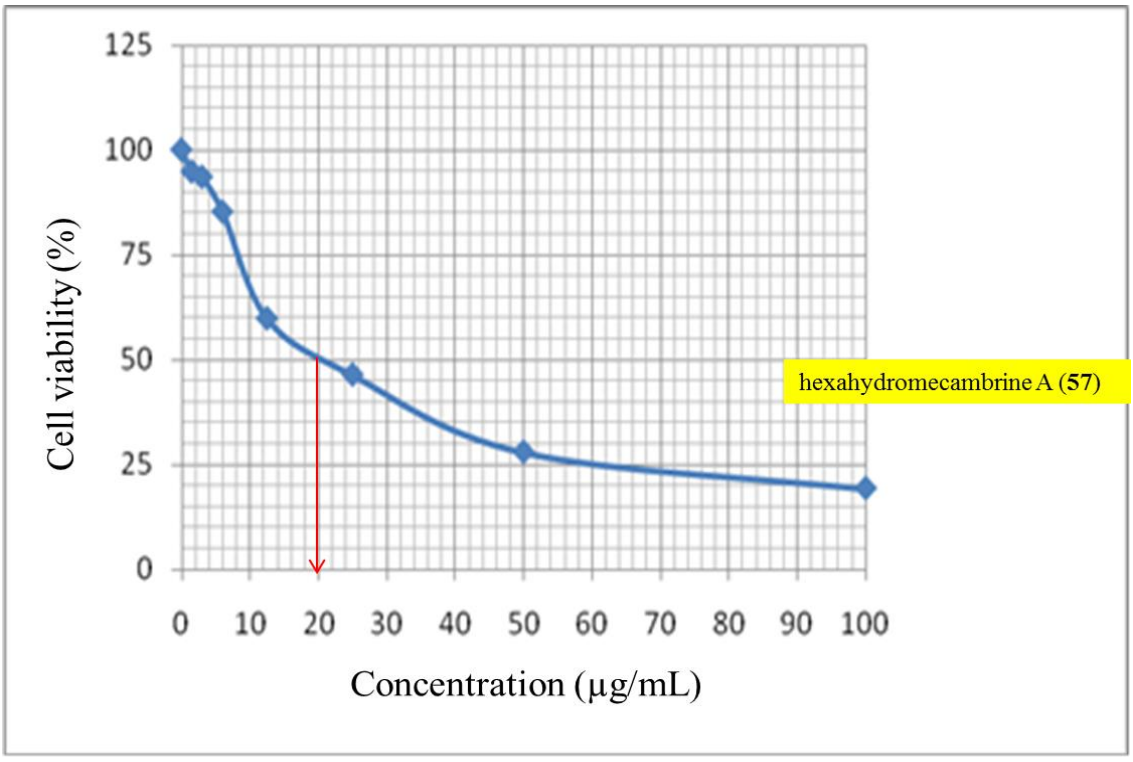
Percentage viability of HepG2 cells treated with different concentration of **lysicamine (54)** with the **IC_{50}= 27 µg/mL** measured after 24 hours using MTT assay.



Percentage viability of HepG2 cells treated with different concentration of **litsericinone (55)** with the **IC_{50}= 14 µg/mL** measured after 24 hours using MTT assay.

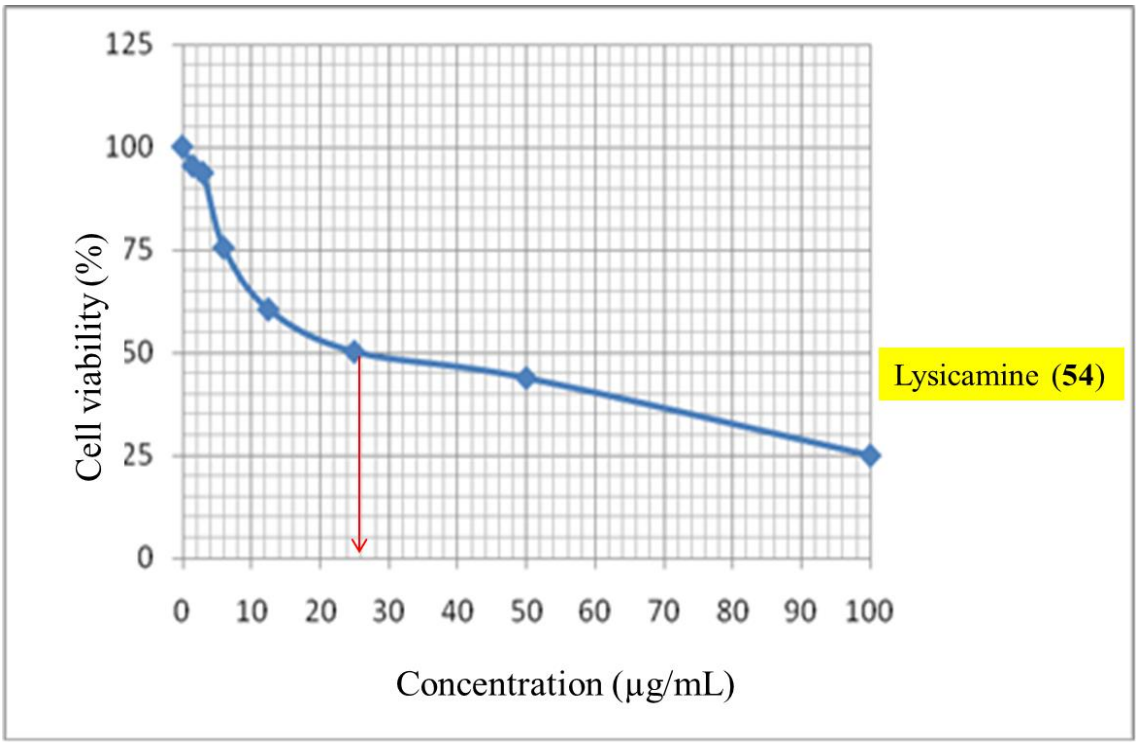


Percentage viability of HepG2 cells treated with different concentration of **8,9,11,12-tetrahydromecambrine (56)** with the **IC_{50}= 81 µg/mL** measured after 24 hours using MTT assay.

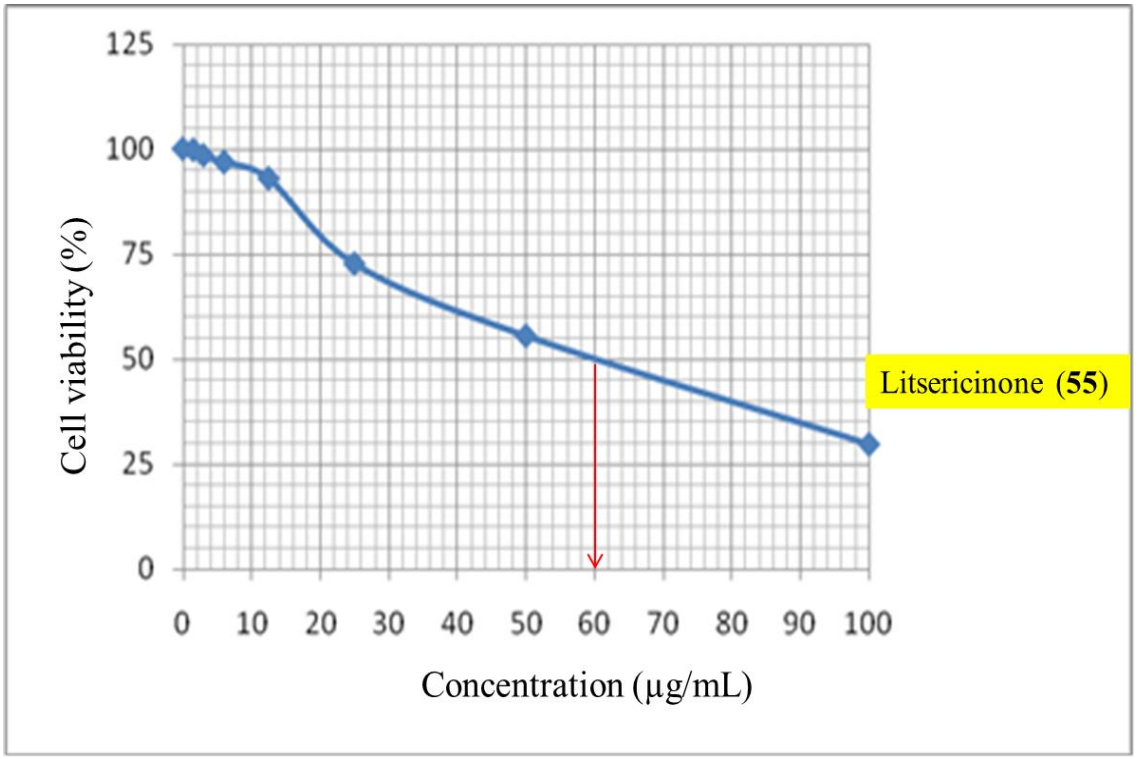


Percentage viability of HepG2 cells treated with different concentration of **hexahydromecambrine A (57)** with the **IC_{50}= 20 µg/mL** measured after 24 hours using MTT assay.

Cytotoxic activity of isolated compounds by using MTT assay against MCF7.



Percentage viability of MCF7 cells treated with different concentration of **lysicamine (54) with the IC_{50}= 26 µg/mL** measured after 24 hours using MTT assay.



Percentage viability of MCF7cells treated with different concentration of **litsericinone (55) with the IC_{50}= 60 µg/mL** measured after 24 hours using MTT assay.

Note: IC_{50} value of **8,9,11,12-tetrahydromecambrine** (**56**) and **hexahydromecambrine A (57)** = >100 that is have no effect on MCF7.